CHIMERIC EXPRESSION PROMOTERS ORIGINATING FROM COMMELINA YELLOW MOTTLE VIRUS
AND CASSAVA VEIN MOSAIC VIRUS

5 **[DESCRIPTION]**

The present invention relates to chimeric expression promoters,
intended in particular for use in the field of plant
biotechnology.

Expression promoters are in general known in the field of
10 biotechnology and genetic manipulation. Insofar as plant
biotechnology is more particularly concerned, the rate of
expression of a gene coding for a polypeptide that it is desired
to produce in a host cell is often dependent on the promoter used.
The problem with this is that the various promoters commonly used
15 are often limited to specific applications or tissues, simply
because of their tissue specificity or expression strength. It is
possible for example to cite the cauliflower mosaic virus promoter
35S as relatively strong promoter, compared to that for example
originating from the the nos gene, these two promoters being more
20 particularly used in the field of plant biotechnology. There thus
exists a need for new promoters that are able to overcome the
disadvantages described above of the application of the promoters
known today.

One attempt at solving this problem has been reported in PCT
25 application published under the number WO 97/48819, which
describes promoters derived from the cassava vein mosaic virus
(CsVMV), all of which comprise a portion of a nucleic acid
sequence having 18 sequential nucleotides of which the homology
to a reference sequence cited in the application is at least 80%.
30 The expressions used in the present description and claims have
the following meaning unless indicated otherwise :
 - "nucleic acid" means DNA or RNA ;
 - "nucleic acid sequence" means a single or double stranded
oligomer or polymer, of nucleotide bases read from the 5' end
35 towards the 3' end, and comprises self-replicating plasmids,
genes, DNA or RNA polymers, infectious or non-infectious, and
functional or non-functional DNA or RNA. In the nucleotide

notation used in the present application, and unless otherwise indicated, the left end of a single stranded nucleotide sequence simple is the 5' end ;

- "derived nucleic acid sequence" means that the sequence derives
5 directly or indirectly from the sequence to which it refers, for example by substitution, deletion, addition, mutation, fragmentation, and/or synthesis of one or more nucleotides ;

- "promoter" or "promoter nucleic acid sequence" means a region of nucleic acid upstream of the start codon for translation which is
10 directly implicated in the recognition and binding of RNA polymerase and other proteins necessary for transcription ;

- "plant promoter" is a promoter capable of initiating transcription in plant cells ;

- "constitutive promoter" is a promoter capable of expressing
15 nucleic acid sequences operably linked thereto, in all or substantially all of the tissues of the host organism throughout the entire development of said organism ;

- "tissue specific promoter" is a promoter capable of selectively expressing, nucleic acid sequences operably linked to said
20 promoter, in certain specific tissues of the host organism ;

- "operably linked to" means the linking of the promoter, to the nucleic acid sequence or gene, coding for a polypeptide to be produced, such that the promoter positively drives transcription of said linked nucleic acid sequence. It should be understood that
25 the promoter sequence also includes transcribed sequences situated between the transcription initiation site and the translation start codon ;

- "expression cassette" means nucleotide sequences capable of directing the expression of a nucleic acid sequence, or a gene,
30 coding for a polypeptide to be produced in a host organism compatible with such sequences. Such expression cassettes comprise at least one promoter and a transcription terminator signal, and optionally other factors necessary or useful for expression ;

- "vector" means expression systems, for example projectiles
35 coated with DNA, nucleic acid based transit vehicles, nucleic acid molecules adapted for delivery of nucleic acid, and circular self-replicating autonomous DNA, for example plasmids, cosmids,

phagemids, etc. If a recombinant micro-organism or cell culture is described as a host for an expression vector, this can also include circular extrachromosomal DNA (such as for example mitochondrial or chloroplast DNA), where DNA has been integrated
5 into the chromosome(s) of the host(s), the vector either being stably replicated with the cells during mitosis as an autonomous structure, integrated into the host genome, or maintained in the nucleus or the cytoplasm of the host ;
   - "plasmid" means a molecule of circular autonomous DNA capable of
10 replication within a cell, and comprises both plasmids called "expression plasmids" and plasmids called "non-expression plasmids". If a recombinant micro-organism or cell culture is described in the present application as the host to an "expression plasmid", this means both molecules of circular extrachromosomal
15 DNA and DNA having been integrated into the host chromosome. If the plasmid is maintained in a cell host, the plasmid is either stably replicated with the cells during mitosis as an autonomous structure, or integrated into the host genome ;
   - "heterologous sequence" or "heterologous nucleic acid sequence"
20 means a sequence originating from a source, or a species, that is foreign to its natural environment, or where it comes from the same environment, has been modified with respect to its original native form. The modification of the nucleic acid sequence can occur for example by treating the nucleic acid with a restriction
25 enzyme to generate a nucleic acid fragment capable of being operably linked to a promoter. The modification can also be carried out using techniques such as site specific or site directed mutagenesis ;
   - "box" means a nucleic acid sequence to which a regulatory
30 function is attributed ;
   - "like" means that the box and/or nucleic acid sequence to which the term refers, comprises a certain sequence identicality or consensus with a known reference box and/or nucleic acid sequence, and preferably of at least 50% sequence identicality, even more
35 preferably a sequence identicality of at least 75%, and most preferably a sequence identicality of at least 90% with the reference sequence. The percentage of sequence identicality is

calculated on the basis of a comparison window of at least 6
contiguous nucleotide bases. The determination of a comparison
window can be made by using sequence alignment algorithms in order
to determine an homology with a reference sequence, for example
5   the local homology algorithm, the homology alignment algorithm,
and the similitude search algorithm, these algorithms also
existing in electronic or computerised form, under the names GAP,
BESTFIT, FASTA et TFASTA. The percentage of sequence identicality
is obtained by comparing the reference sequence with the box
10 and/or nucleic acid sequence ;
- "situated" means the position on a nucleic acid sequence of an
identified element, such as a "box", a restriction enzyme site, or
a codon having a particular function. The position given is
indicated with a number that refers to the position of the start
15 of the element in the nucleic acid sequence, in the reading fram
direction of the latter, that is to say, most frequently, and
unless otherwise indicated from 5' to 3' ;
- "transgenic plant" means a plant having been obtained using
genetic manipulation techniques, and covers whole plants obtained
20 thereby, their progeny, as well as vital plant organs, for example
roots, stems and leaves, obtained using these techniques. The
transgenic plants according to the present invention can have
different levels of ploidy, and can in particular be polyploid,
diploid, and haploid ;
25 - "propagule" means a structured or unstructured collection or
assembly or association of plant cells, from which it is possible
to regenerate a whole plant, for example explants, calli, stems,
leaves, roots, cuttings, and even seeds.
The applicant of the present invention has taken a different
30 approach to that taken by the applicant of the previously
discussed PCT patent application. Indeed, the present applicant
has serendipitously succeeded in producing chimeric promoters
capable of satisfying and fulfilling the need described
previously, and especially capable of increasing the rate of
35 expression of a gene or nucleic acid sequence coding for a
polypeptide to be produced, in a host cell, and more particularly
in a plant cell or regenerated plant, with respect to the existing

promoters most often used. Furthermore, the applicant has also succeeded in producing a complete family of promoters in order to be able to choose the promoter most suited to the envisaged task or application and the environment in which it will be put to

5 work, and thus making it possible to control to a certain extent, the expression rate of a gene to be expressed coding for a polypeptide to be produced.

Consequently, one of the objects of the present invention is a chimeric expression promoter comprising at least one nucleic acid

10 sequence, derived from a first plant promoter comprising a plant vascular expression promoter region, said plant vascular expression promoter region being replaced with a nucleic acid sequence derived from a second plant promoter and comprising a plant green tissue expression promoter region.

15 Preferably, the first plant promoter originates from the Commelina Yellow Mottle Virus (CoYMV) and the second plant promoter originates from the Cassava Vein Mosaic Virus (CsVMV). Even more preferably, the promoter nucleic acid sequences originate from the intergenic regions of the first and second

20 promoters.

In a particularly preferred embodiment, the chimeric expression promoter comprises at least a part of a nucleic acid sequence identified under the number SEQ.ID01 fused to at least a part of of a nucleic acid sequence identified under the number SEQ.ID02.

25 In an even more preferred embodiment, the chimeric promoter according to the invention is selected from the group consisting of the nucleic acid sequences identified under the numbers SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06 and SEQ.ID07.

According to another object of the present invention, the

30 applicant has discovered that it was possible to produce particularly active chimeric expression promoters by starting from a base promoter of viral origin, of which a part consists of an exogenic element that is capable of promoting plant green tissue expression (GT). Preferably, the exogenic GT promoter element is

35 also of viral origin. Furthermore, and according to a preferred embodiment of this aspect of the invention, the promoter of viral origin originates from the Commelina Yellow Mottle Virus (CoYMV).

Preferably, the exogenic promoter element originates from the Cassava Vein Mosaic Virus (CsVMV). Even more preferably, the exogenic GT element replaces the endogenic element capable of promoting vascular tissue expression (VT) of the promoter of viral

5 origin.

According to a preferred embodiment of the previously defined objects of the invention recited above, the chimeric promoters further comprise at least one "endosperm like" box, and more preferably from 4 to 10 "endosperm like" boxes, and most

10 preferably 6 "endosperm like" boxes.

According to another preferred embodiment, the promoters further comprise at least one "as1 like" box operably linked to the plant green tissue GT promoter element.

According to yet another preferred embodiment, the promoters of

15 the present invention comprise at least one "as1" box operably linked to the plant green tissue (GT) promoter element.

In another preferred embodiment, the promoters further comprise at least one "as2" box operably linked to the plant green tissue (GT)promoter element.

20 Preferably, the one or more of the "as1 like", "as1", and "as2" boxes are operably linked upstream or downstream of the plant green tissue expression GT promoter element.

Even more preferably, the one or more of the "as1 like", "as1", and "as2" boxes are operably linked in normal (5'>3') or inverse

25 (3'>5') orientation.

Most preferably, the promoter comprises at least one "as2/as2/as2" box in normal (5'>3') or inverse (3'>5') orientation.

Preferably, the previously described promoter is selected from the group consisting of the nucleic acid sequences identified under

30 the numbers SEQ.ID01, SEQ.ID02, SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07, SEQ.ID19, SEQ.ID20, SEQ.ID21, SEQ.ID22, SEQID.23, SEQ.ID24, and SEQ.ID25.

Another object of the present invention is an expression cassette comprising at least one nucleic acid sequence, derived from a

35 first plant promoter comprising a plant vascular expression promoter region, said plant vascular expression promoter region being replaced with a nucleic acid sequence derived from a second

plant promoter and comprising a plant green tissue expression
promoter region, the promoter nucleic acid sequences being
operably linked to a nucleic acid sequence, or gene, to be
expressed coding for a polypeptide to be produced, the latter
5 nucleic acid sequence or gene being operably linked to a
transcription terminator nucleic acid sequence.
Preferably, and according to this embodiment, said first plant
promoter originates from the Commelina Yellow Mottle Virus (CoYMV)
and said second plant promoter originates from the Cassava Vein
10 Mosaic Virus (CsVMV). Even more preferably, the expression
cassette comprises at least a part of a nucleic acid sequence
identified under the number SEQ.ID01 fused to at least a part of a
nucleic acid sequence identified under the number SEQ.ID02.
Even more preferably, the nucleic acid sequence of the chimeric
15 promoter of the expression cassette is selected from the group
consisting of the sequences identified under the numbers SEQ.ID03,
SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07, SEQ.ID19, SEQ.ID20,
SEQ.ID21, SEQ.ID22, SEQID.23, SEQ.ID24, and SEQ.ID25.
In accordance with another object of the present invention, there
20 is provided an isolated promoter nucleic acid sequence, selected
from the group consisting of the sequences identified under the
numbers SEQ.ID03, SEQ.ID04, SEQ.ID05, SEQ.ID06, SEQ.ID07,
SEQ.ID19, SEQ.ID20, SEQ.ID21, SEQ.ID22, SEQID.23, SEQ.ID24, and
SEQ.ID25.
25 Yet another object of the invention relates to desoxynucleotide
building blocks for the production of promoters or promoter
nucleic acid sequences as defined above. These building blocks can
be :
    - "directional" building blocks, that is to say sequences
30 that read in the same direction as the reading frame of the final
promoter sequence, usually from the 5' end to the 3' end; and/or
    - "guide" blocks, that is to say sequences of whose ends
comprise nucleotide bases that overlap with the ends of the
directional building blocks.
35 In this way, and preferably, the directional building block
corresponds to at least one sequence selected from the group
consisting of the sequences identified under the numbers SEQ.ID08,

SEQ.ID09, SEQ.ID10, SEQ.ID11, SEQ.ID13, and SEQ.ID14.
Moreover, it is preferred to use desoxynucleotide "guide"
corresponding to at least one sequence selected from the group
consisting of the sequences identified under the numbers SEQ.ID15,
5 SEQ.ID16, SEQ.ID17, and SEQ.ID18.
Yet another object of the present invention is a vector comprising
a promoter, or a promoter nucleic acid sequence, capable of
initiating transcription of a nucleic acid sequence, or gene,
coding form a polypeptide to be produced, wherein the promoter or
10 promoter nucleic acid sequence corresponds to a chimeric
expression promoter or to a promoter nucleic acid sequence as
described previously above.
Preferably, the vector is selected from the group consisting of
the binary vectors pMRT1152, pMRT1171, pMRT1172, pMRT1185,
15 pMRT1186, pMRT1187, pMRT1188, pMRT1182, pMRT1245, pMRT1246,
pMRT1247, pMRT1248, pMRT1249, pMRT1250, pMRT1251, pMRT1252,
pMRT1253 and pMRT1254.
Finally, another object of the present invention is a process for
the manufacture of a chimeric expression promoter or an isolated
20 promoter nucleic acid sequence as described previously, wherein
said process comprises the steps consisting of :
        - carrying out a ligation chain reaction, called LCR, to
produce single stranded continuous DNA from at least one
desoxynucleotide building block selected from the group consisting
25 of the "directional" desoxynucleotide building blocks S1, S2, S3,
S4, S5, S6, and S7 identified under the numbers SEQ.ID08,
SEQ.ID09, SEQ.ID10, SEQ.ID11, SEQ.ID12, SEQ.ID13 and SEQ.ID14
respectively, and at least one "guide" desoxynucleotide building
block for said promoter nucleic acid sequence or promoter selected
30 from the group consisting of the guide desoxynucleotides G1, G2,
G3 and G4 identified under the numbers SEQ.ID15, SEQ.ID16,
SEQ.ID17 et SEQ.ID18 respectively ;
        - carrying out PCR amplification on the single stranded DNA
obtained from the previous step to produce a double stranded DNA
35 corresponding to the chimeric expression promoter or the promoter
nucleic acid sequence ;
        - optionally isolating the promoter of promoter nucleic acid

sequence.

Advantageously, and preferably, the desoxynucleotide building blocks are phosphorylated before ligation. Even more preferably, the ligation is carried out in the presence of at least one DNA ligase in a thermocycle, under the following conditions :

- a cycle of about one minute at about 94°C ;

- eight identical cycles, each one consisting of the following steps :

- one minute at 65°C, one minute at 57°C, one minute at 52°C, one minute at 48°C, one minute at 43°C and ten minutes at 37°C.

Yet another object of the present invention is a transgenic plant having stably integrated into its genome at least one promoter or at least one promoter nucleic acid sequence as defined previously. Preferably, the transgenic plant is selected from dicotyledonous species, and preferably potato, tobacco, cotton, lettuce, tomato, melon, cucumber, pea, rape, canola, beetroot, or sunflower, or from monocotyledonous species, and preferably wheat, barley, oat, rice, or corn.

Yet still another object of the present invention, is a propagule of a transgenic plant as defined previously, preferably the propagule is a seed.

According to the present invention, another object is a cell containing a promoter or promoter nucleic acid sequence as defined above, and preferably, the cell is a plant cell.

According to another object of the present invention, a method of expression of a nucleic acid sequence, or gene, is provided, that codes for a polypeptide to be produced, by the cell, wherein the method comprises the steps consisting of :

- transforming a cell with a vector comprising at least one promoter or at least one promoter nucleic acid sequence as defined previously ;

- culturing the cell under conditions enabling expression of the nucleic acid sequence, or gene, coding for said polypeptide and production thereof. Preferably, the cell is a prokaryotic or eukaryotic cell, and more preferably is selected from the group consisting of microbial cells, algal and microalgal cells, fungal cells, insect cells, animal cells, mammalian and human cells, and

plant cells, and most preferably is a plant cell.

According to yet another object of the present invention, a method of manufacture is supplied for manufacturing a transgenic plant or a propagule wherein the method comprises the steps consisting of :

5   - transforming a plant cell with a vector comprising at least one promoter or at least one promoter nucleic acid sequence as defined previously ;

        - selecting the plant cell having integrated the promoter or promoter nucleic acid sequence ;

10   - propagated the selected and transformed plant cell, by culture, or by regeneration of whole chimeric or transgenic plants.

BRIEF DESCRIPTION OF THE FIGURES

The invention will be better understood through the following
15 detailed description of one or more preferred embodiments, given purely as non-limiting examples, and with referral to the annexed drawing in which :

        - Figures I, II and III schematically represent the structures of the comparative reference constructs, enabling a
20 comparison of the chimeric promoters of the present invention with those already known and used. In Figure I, the construction concerned contains the reporter gene coding for ß-glucuronidase in the total absence of any promoter sequence as such, and thus useful as a negative control.

25   - Figure II schematically represents a construct containing the ß-glucuronidase gene under the control of the CaMV double 35S promoter, useful as a strong reference control ;

        - Figure III represents a construct useful as an internal reference for the transient expression experiments, and includes
30 the reporter gene coding for a luciferase under the control of the CaMV 35S promoter ;

        - Figure IV schematically represents the structure of several preferred embodiments of chimeric promoters produced in accordance with the present invention. The chimeric promoters MPr1116 and
35 MPr1117 were obtained using the technique called 1b-PCR. MPr1146 and MPr1147 were obtained by cloning the activator elements as1 and as2 from the CaMV promoter at the restriction enzyme site

DraIII. The promoter MPr1154 was obtained through deletion of the two "as-1 like" sequences from the CoYMV promoter present in the 5' region of MPr1147. All of these promoters were cloned at the restriction sites PstI and BamHI into the vector pMRT1144 in order
5 to obtain transcriptional fusion with the reporter gene *uidA* ;

- Figure V represents histochemical staining of tobacco leaves transformed with different promoters in accordance with the present invention. The tobacco leaves were transformed by a biolistic method using a « PDS1000/He » gene gun available from
10 BIORAD, under the following conditions : split rupture disks at 900 psi, 2 µg of DNA bombarded over two successive firings, projectiles consisting of gold beads or spheres of about 1 µm in diameter, plant material positioned at 6 then 9 cm from the macrocarrier. After bombardment, the leaves were incubated in the
15 dark in a culture chamber for 48 hours to enable expression of the reporter gene. The leaves were then incubated in a 0.1 M phosphate buffer containing 2 mg/ml of X-Glu at 37°C for 24 to 48 hours, then bleached in a 70% ethanol bath.

- Figure VI represents a graph comparing the relative
20 promoter activity of the different constructs after transient expression in tobacco leaves. Three days after bombardment the leaves were ground then the crude extract clarified by centrifugation. The ß-glucuronidase and luciferase activities were measured using fluorimetric methods on crude extract aliquots,
25 then the ratio of GUS activity / LUC activity was determined. The histograms correspond to the average of the ratios for a given construct +/- standard mean error ;

- Figure VII schematically represents other preferred embodiments of chimeric promoters according to the present
30 invention, where :

the dark disk-shaped symbols represent the green tissue expression specific element ;

the small white parallelepiped symbols represent the "endosperm like" boxes ;

35 the small and large black hatched parallelepipeds represent respectively the "as-2" and "as-1" boxes from the CaMV promoter.

- Figure VIII represents a comparison of the relative

activity of the different promoters of the invention in transient expression experiments in corn albumen, where the β-glucuronidase and luciferase activities were measured by fluorimetry on an aliquot of crude extract. The histograms correspond to the average

5 for a given construction +/- standard mean error ;

   – Figure IX represents a comparison of the relative activity of the chimeric promoters MPr1116, MPr1146, MPr1167 and reference promoter MPr1092, evaluated in stable tobacco expression. Samples were taken from each primary transformant at 2, 4, 6, 8, and 10

10 weeks after transfer of the plants into the greenhouse. The β-glucuronidase activity was measured on each sample and weighted in relation to the total quantity of total protein. For each series of transformants, at a given time, the activities are classed in decreasing order and compared ;

15   – Figure X·represents a comparison of the relative activity of the chimeric promoters MPr1162, MPr1164, MPr1165, MPr1167 and the reference promoter MPr1092, evaluated in stable tobacco expression. Samples were taken from each primary transformant at 2, 4, 6, 8, and 10 weeks after transfer to the greenhouse. The

20 β-glucuronidase was measured on each sample and weighted in relation to the total quantity of protein. For each series of transformants, at a given times, the activities were classed in decreasing order and compared.

In the various figures, certain terms have the following

25 meanings :

   – uidA = the sequence coding for ß-glucuronidase ;

   – IV2 = the patatin gene intron ;

   – nos term = the terminator from the Nopaline Synthase gene ;

   – 35S term = the RNA 35S CaMV terminator ;

30   – CaMV = the cauliflower mosaic virus ;

   – as-1 = activating sequence 1 from the CaMV 35S promoter ;

   – as-2 = activating sequence 2 from the CaMV 35S promoter ;

   – B = the endonuclease restriction site BamHI ;

   – E = the endonuclease restriction site EcoRI ;

35   – H = the endonuclease restriction site HindIII ;

   – P = the endonuclease restriction site PstI ;

   – Sp = the endonuclease restriction site SphI.

- D = the endonuclease restriction site DraIII ;
- N = the endonuclease restriction site NdeI ;
- S = the endonuclease restriction site SpeI ;
- CoYMV = the Commelina Yellow Mottle Virus ;
5 - CsVMV = the Cassava Vein Mosaic Virus ;
- TATA = the TATA box;
- +1 = the transcription initiation site ;
- "like" means that the sequence is not 100% homologous to the
sequence to which it refers, as defined previously.
10

EXAMPLES

Example 1

Comparative Constructs (controls)

In order to enable the comparison between the chimeric promoters
15 of the present invention, and those known and currently used, the
uidA gene coding for β-glucuronidase (Jefferson et al., 1986) and
containing the intron IV2 sequence from the potato patatin gene
ST-LS1 (Vancanneyt et al., 1990) (uidA-IV2) was placed under the
control of one of the promoters and the terminator from the
20 nopaline synthase gene (nos term) from Agrobacterium tumefaciens
into the plasmid pGEM3Z commercialised by Promega Corp. (Madison,
USA).

1.1. Construction of negative control pMRT1144.

In order to facilitate cloning, a plasmid derived from pGEM3Z,
25 containing only the sequences "uidA-IV2/nos term" and lacking any
promoter sequence was produced. This plasmid was designated
pMRT1144 and served as the negative control (Fig. I).
In order to insert the uidA/nos term sequence into pGEM3Z, the
uidA sequence under the control of the entire promoter from the
30 pea plastocyanin gene and the nopaline synthase terminator, were
isolated from 5 µg of plasmid pGA492-PpetE. This plasmid had been
obtained by cloning, into the plasmid pGA492-Pem2-uidA, the petE
promoter originating from the pea plastocyanin gene from the
plasmid pKHn2 (Pwee and Gray, 1993) instead of the em2 promoter
35 (Gaubier et al., 1993), originating from the plasmid bp
I221-Pem2. The plasmid bp I221-Pem2 was digested with 20 units of
each of the enzymes HindIII and EcoRI for 1 hour at 37°C. Then,

the expression cassette "P*em2*/*uidA*/*nos* term" was separated by
electrophoresis on 0.8% agarose gel, electroeluted, precipitated
in the presence of 1/10 volume 3M sodium acetate pH 4.8 and 2.5
volumes of absolute ethanol at -80°C for 30 minutes, centrifuged
5   at 12000 g for 30 min, washed in 70% ethanol, dried, resuspended
in water and inserted at the HindIII and EcoRI sites of the
plasmid pGA492 (An, 1986) previously digested by these two enzymes
for 1 h at 37°C, precipitated in the presence of 1/10 volume 3M
sodium acetate pH 4.8 and 2.5 volumes absolute ethanol at -80°C
10 f.r 30 min, centrifuged at 12000 g for 30 min, washed in 70%
ethanol, dried and then resuspended in water. Ligation was carried
out in the presence of 1.0 µl T4 10X DNA ligase buffer (Amersham)
and 2.5 unites of T4 DNA ligase (Amersham) at 14°C for 16 h.
Previously prepared viable and competent *Escherichia coli* DH5α,
15 bacteria were transformed (Hannahan, 1983). The plasmid DNA of the
obtained clones, selected on Luria-Bertani media (LB,
bactotryptone 10 g/l, yeast extract 5 g/l, NaCl 10 g/l, Agar 15
g/l) supplemented with tetracycline (12 mg/l), was extracted
according to the alkaline lysis method (Birnboim et Doly, 1983)
20 and analysed by enzymatic digestion.
Starting from the obtained pGA492-P*em2*-*uidA* plasmid, the promoter
P*em2* was deleted by double digestion with HindIII and XbaI. The
plasmid fragment was separated by electrophoresis on 0.8% agarose
gel, electroeluted, precipitated in the presence of 1/10 volume 3M
25 sodium acetate pH 4.8 and 2.5 volumes absolute ethanol at -80°C
for 30 min, centrifuged at 12000 g for 30 min, washed in 70%
ethanol, dried, subjected to a Klenow fragment of DNA polymérase I
(New England Biolabs) for 30 min at 37°C according to the
manufacturer's recommendations. Then, it was deproteinized by
30 extraction a volume of phenol, then a volume of phenol :
chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally a volume
of chloroform : isoamyl alcohol (24:1 v/v), precipitated in the
presence of 1/10 volume 3M sodium acetate pH 4,8 and 2,5 volumes
absolute ethanol at -80°C for 30 min, then centrifuged at 12000
35 g for 30 min, washed in 70% ethanol, dried, resuspended in water.
Then, it was dephosphorylated for 1 h at 37°C using 10 units of
calf intestine alkaline phosphatase (Boehringer Mannheim)

according to the manufacturer's recommendations, deproteinized by extraction with a volume of phenol, then a volume of phenol : chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally a volume of chloroform : isoamyl alcohol (24:1 v/v), precipitated in the

5 presence of 1/10 volume 3M sodium acetate pH 4.8 and 2.5 volumes absolute ethanol at -80°C for 30 min then centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried then resuspended in water. The resulting plasmid was designated pGA492ΔP*em2*.

In parallel, the promoter *petE* (818 bp) which corresponds to the

10 promoter of the pea plastocyanin gene, was obtained from the plasmid pKHn2 by digestion with NcoI for 1 h at 37°C. The 828 bp promoter fragment was isolated on 0.8% agarose gel, electroeluted, precipitated in the presence of 1/10 volume 3M sodium acetate pH 4.8 and 2.5 volumes absolute ethanol at -80°C for 30 min,

15 centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried, resuspended in water, then subjected to the action of 5 units of Mung Bean nuclease (New England Biolabs) for 30 min at 30°C according to the manufacturer's recommendations , deproteinized by extraction with a volume of phenol, then a volume of phenol :

20 chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally a volume of chloroform : isoamyl alcohol (24:1 v/v), precipitated in the presence of 1/10 volume 3M sodium acetate pH 4.8 and 2.5 volumes absolute ethanol at -80°C for 30 min then centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried then resuspendu in water.

25 This promoter fragment was inserted into the plasmid pGA492ΔP*em2,* described above, in the presence of 1.0 μl T4 10X DNA ligase buffer (Amersham) and 2.5 units of T4 DNA ligase (Amersham) at 14°C for 16 h. Previously prepared viable and competent *Escherichia coli* DH5α bacteria were transformed. The plasmid DNA of the obtained

30 clones, selected on LB media supplemented with tetracycline (12 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion . The resultant plasmid was designated pGA492-*petE* prom.

In order to isolate the expression cassette "*petE* prom / *uidA* /

35 *nos* term", 5 μg of plasmid pGA492-*petE* prom were digested with PstI (site situated in the 5' region of the promoter of the pea

plastocyanin gene) and EcoRI (site situated in the 3' region of the terminator sequence) for 1 h at 37°C, subjected to 0.8 agarose gel electrophoresis and purified on a QIAquick affinity column (Qiagen, Hilden, Germany) according to the recommendation of the

5 supplier. Furthermore, 500 ng of the plasmid pGEM3Z were simultaneously digested for 1 h at 37°C with EcoRI and PstI (restriction sites present in the multiple cloning site or polylinker), subjected to 0.8% agarose gel electrophoresis, then purified on a QIAquick affinity column.

10 The ligation was carried out with 50 ng of vector pGEM3Z-PstI/EcoRI and 50 ng of the expression cassette *petE* prom / *uidA* / *nos* term for 1 night at 18°C in a reaction volume of 12 μl in the presence of 1.2 μl T4 10X DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs).

15 Previously prepared Escherichia coli DH5α bacteria were transformed with the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion. The plasmid

20 obtained was designated pGEM3Z-*petE* prom.

In order to insert the 192 bp IV2 intron from the potato patatin gene into the coding *uidA* sequence, an internal portion of this gene (fragment SnaBI / BstI of 710 bp in pGEM3Z-*petE* prom) was excised then replaced with the equivalent sequence containing the

25 IV2 intron (fragment SnaBI / BstI of 902 bp). In order to achieve this, the plasmid pGEM3Z-*petE* prom was digested for 1 h at 37°C with SnaBI (restriction site situated at position +383 bp downstream of the initiator ATG codon of the *uidA* gene) then for 1 h at 65°C with BstI (site situated at position +1093 bp). The

30 plasmid deleted of this 710 bp fragment was isolated by 0.8% gel agarose electrophoresis, then purified on a QIAquick affinity column. The fragment BstBI/SnaBI of 902 bp corresponding to the IV2 intron sequence followed by the *uidA* sequence stretching from position +383 to +1093 bp, was isolated and purified from the

35 plasmid pSCV1.2-GI. This plasmid derives from the plasmid pSCV1.2 which in turn derives from the plasmid pSCV1 constructed by G.A. Edwards in 1990 according to the usual cloning methods. The binary

plasmid pSCV1.2 was obtained through cloning of the fragment
HindIII bearing the expression cassette "35S prom / *nptII* / *nos*
term" (Fromm *et al.*, 1986) at the HindIII site of pSCV1. The
expression cassette "35S prom / GUS-IV2 / 35S term" was obtained
5 by digesting the plasmid p35S GUS INT with HindIII for 1 h at 37
°C as described by Vancanneyt *et al.* (1990). The DNA fragment
corresponding to the expression cassette was isolated on 0.8 %
agarose gel, electroeluted then precipitated in the presence of
1/10 volume 3M sodium acetate pH 4.8 and 2.5 volumes absolute
10 ethanol at -80°C for 30 min then centrifuged at 12000 g for 30
min, washed in 70 % ethanol, dried and resuspended in water. The
5' protruding ends of this fragment were blunted by the action of
the DNA polymerase I Klenow fragment (New England Biolabs) for 30
min at 37°C according to the manufacturer's recommendations , and
15 the fragment was deproteinized by extraction with a volume of
phenol, then a volume of phenol : chloroform : isoamyl alcohol
(25:24:1 v/v/v) and finally a volume of chloroform : isoamyl
alcohol (24:1 v/v), precipitated in the presence of 1/10 volume 3M
sodium acetate pH 4,8 and 2,5 volumes absolute ethanol at -60°C
20 for 30 min then, centrifuged at 12000 g for 30 min, washed in 70%
ethanol, dried and finally ligated with 20 ng of plasmid pSCV1.2,
digested with SmaI for 1 h at 25°C, in the presence of 1.0 µl T4
10X DNA ligase buffer (Amersham) and 2.5 units of T4 DNA ligase
(Amersham) at 14°C for 16 h. Previously prepared competent and
25 viable *Escherichia coli* DH5α bacteria were transformed. The plasmid
DNA of the obtained clones, selected on LB media supplemented
with ampicillin (50 mg/l), was extracted according to the
alkaline lysis method and analysed by enzymatic digestion .
Five micrograms (5 µg) of the plasmid pSCV1.2-GI were digested for
30 1 h at 37°C with SnaBI (restriction site situated at position +383
bp downstream of the initiator ATG codon of the gene *uidA*) then
for 1 h at 65°C with BstBI (site situated at position +1285 bp).
The 902 bp fragment was isolated by 1.0% agarose gel
electrophoresis, then purified on a QIAquick affinity column .
35 The ligation was carried out with 20 ng of vector pGEM3Z-*petE* prom
BstBI/SnaBI and 80 ng of the 902 bp fragment BstBI/SnaBI, for 1
night at 18°C in a reaction volume of 10 µl in the presence of 1.0

μl of T4 10X DNA ligase buffer (New England Biolabs) et 400 units
of T4 DNA ligase (New England Biolabs). Previously prepared
competent and viable Escherichia coli DH5α bacteria were
transformed with half of the ligation reaction mixture. The
5 plasmid DNA of the obtained clones, selected on LB media
supplemented with ampicillin (50 mg/l), was extracted according to
the alkaline lysis method and was analysed by enzymatic digestion.
The plasmid obtained was designated pGEM3Z-petE prom / IV2.
In order to eliminate the promoter sequence corresponding to the
10 818 bp fragment (petE) from the plasmid pGEM3Z-petE prom / IV2,
the latter was digested for 1 h at 37°C with BamHI then, for 1 h
at 37°C with PstI, isolated by electrophoresis on 0.8% gel
agarose, then purified on a QIAquick affinity column. The
protruding 5' ends of this plasmid were blunted with Pfu DNA
15 polymerase (Stratagene, La Jolla, USA) according to the
supplier's recommendations. The ligation was carried out with 10
ng of thus modified plasmid for 1 night at 18°C in a reaction
volume of 12 μl, in the presence of 1.2 μl of T4 10X DNA ligase
buffer (New England Biolabs) and 400 units of T4 DNA ligase (New
20 England Biolabs). Previously prepared competent and viable
Escherichia coli DH5α bacteria were transformed with half the
reaction mixture of ligation. The plasmid DNA of the obtained
clones, selected on LB media supplemented with ampicillin (50
mg/l), was extracted according to the alkaline lysis method,
25 analysed by enzymatic digestion, et verified by sequencing
according to the method described by Sanger et al. (1977). The
plasmid obtained was designated pMRT1144 (Fig. I).

1.2. Construction of the positive control MPr1092.

In order to obtain a reference promoter sequence, the "double 35S"
30 promoter from the cauliflower mosaic virus (CaMV D35S prom), was
placed upstream of the sequence uidA-IV2/nos term. The plasmid
pMRT1092 (Fig. I) resulted from the following cloning steps :
First of all, the 192 bp IV2 intron from the potato patatin gene
was inserted into the coding sequence uidA at position +383 bp as
35 described in section 1.1. A one microgram amount (1 μg) of plasmid
pBI221 (Clontech, CA, USA) was digested for 1h30 at 37°C with

SnaBI then for 1h30 at 65°C with BstBI. The plasmid deleted of the
710 bp fragment was isolated by 0.8% agarose gel electrophoresis ,
then purified on a QIAquick affinity column.

A twenty nanogram amount (20 ng) of the vector pBI221 BstBI/SnaBI
and 80 ng of the 902 bp fragment BstBI/SnaBI originating from
pSCV1.2-GI as described previously, were ligated for 1 night at
18°C in a reaction volume of 10 µl, in the presence 1 µl of T4 10X
DNA ligase buffer (New England Biolabs) and 400 units T4 DNA
ligase (New England Biolabs). Previously prepared competent and
viable Escherichia coli DH5α bacteria were transformed with half
of the ligation reaction mixture. The DNA of the obtained clones,
selected on LB media supplemented with ampicillin (50 mg/l), was
extracted according to the alkaline lysis method and analysed by
enzymatic digestion. The plasmid obtained was designated
pBI221/uidA-IV2.

Next, the sequence of the simple 35S CaMV promoter present in the
plasmid pBI221/uidA-IV2 was replaced with the sequence "CaMV
D35S". In order to achieve this, the plasmid pBI221/uidA-IV2 was
digested for 10h30 at 37°C with 10 units of HindIII, then the
sticky ends were blunted by the action of the Klenow fragment of
DNA polymerase I (New England Biolabs) for 30 min at 37°C
according to the manufacturer's recommendations. After
purification of the product of this reaction on a QIAquick
affinity column, the DNA was digested for a night at 37°C with 10
units of BamHI. The plasmid fragment, corresponding to the vector
deleted of the 828 bp CaMV 35S promoter fragment, was isolated by
0.8% agarose gel electrophoresis, then purified on a QIAquick
affinity column .

The CaMV D35S promoter was obtained from the plasmid pJIT163Δ.
This plasmid derives from pJIT163 which in turn derives from the
plasmid pJIT160 (Guérineau and Mullineaux, 1993). The plasmid
pJIT163 possesses a ATG codon between the sites HindIII and SalI
of the polylinker. In order to delete this ATG and obtain the
plasmid pJIT163Δ, the plasmid DNA of pJIT163 was digested with
HindIII and SalI, purified by 0.8% agarose gel electrophoresis ,
electroeluted, precipitated in the presence of 1/10 volume 3M
sodium acetate pH 4.8 and 2.5 volumes absolute ethanol at −80°C

for 30 min, centrifuged at 12000 g for 30 min, washed in 70% ethanol , dried, subjected to the action of the Klenow fragment of DNA polymerase I (New England Biolabs) for 30 min at 37°C according to the manufacturer's recommendations, deproteinized by extraction with a volume of phenol, then a volume of phenol : chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally a volume of chloroform : isoamyl alcohol (24:1 v/v), precipitated in the presence of 1/10 volume 3M sodium acetate pH 4.8 and 2.5 volumes absolute ethanol at -80°C for 30 min, then centrifuged at 12000 g for 30 min, washed in 70% ethanol, dried and finally ligated in the presence of 1.0 µl of T4 10X DNA ligase buffer (Amersham) et 2.5 units T4 DNA ligase (Amersham) at 14°C for 16 h. Previously prepared viable and competent *Escherichia coli* DH5α bacteria were transformed. The plasmid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion.

Ten micrograms (10 µg) of the plasmid pJIT163Δ were digested for 10h30 at 37°C with 10 units of KpnI (site situated in the 5′ region of the promoter) then the sticky ends were blunted with the action of 6 units of T4 DNA polymerase (New England Biolabs) for 30 min at 37°C according to the manufacturer's recommendations . After purification of the product of this reaction on a QIAquick affinity column, the DNA was digested for a night at 37°C with 10 units of BamHI. The resulting 761 bp DNA fragment, corresponding to the D35S promoter was isolated by 1.0% agarose gel electrophoresis, then purified on a QIAquick affinity column . The reaction mixture containing 10 ng of plasmid vector, 100 ng of the 761 bp fragment, 1.0 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units T4 DNA ligase (New England Biolabs) was subjected to ligation in 10 ml for a night at 18°C. Previously prepared viable and competent Escherichia coli DH5α bacteria were transformed with half of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion.

The plasmid obtained was designated pMRT1092 (Fig. II).

1.3. Description of the reference plasmid pCaMV35Sluc.

The plasmid used as an internal reference for transient expression was pCaMV35Sluc (Torrent et al., 1997) which contains the expression cassette for the luciferase (luc) reporter gene under the control of the RNA CaMV 35S promoter and terminator (Fig. III).

Example 2

Construction of chimeric promoters combining elements from the CsVMV and CoYMV promoters.

The entire promoter of the intergenic region of CoYMV corresponds to a sequence of 1038 bp stretching from position -1026 bp to position +12 bp (with respect to the 5' end of the CoYMV transcript, and previously identified as sequence EMBL X52938, by Medberry et al., 1992). A fragment of 243 bp of this whole promoter (Medberry and Olszewski, 1993) was retained for the construction of the chimeric promoters according to the present invention as identified in the sequence listing under SEQ.ID01. On this 243 bp fragment, several potentially regulatory sequences have been putatively identified (from the 5' end in the direction of the 3' end, the positions of which are indicated in in base pairs (bp) with respect to the transcription initiation start point +1 as illustrated in Figure IV:

- an "as-1 like" box of 16 bp in length having a certain similarity to the activating sequence 1 (as-1) which is present in the CaMV 35S promoter, and stretching from position -226 bp to position -210 bp ;

- a 76 bp sequence responsible for the expression in vascular tissues (Medberry et al., 1992), stretching from position -161 bp to position -85 bp, and designated VT in Figure IV ;

- a GTAA element specific to expression in green tissue situated at position -76 bp ;

- three "endosperm like" boxes having a certain similarity to the boxes responsible for specific expression in the endosperm of cereal plants, located at positions -118 bp, -77 bp and -20 bp ;

- a "TATA" box, at position -32 bp;

- the start point of transcription +1 (position 1);

- a 5' untranslated region (UTR) stretching from position +1 to position +12 bp .

The promoter of the intergenic region of CsVMV corresponds to a sequence of 515 bp stretching from position +7162 bp to position +7677 bp (previously identified as sequence EMBL U59751 , Verdaguer et al., 1996). On this 515 bp fragment, identified in the sequence listing under SEQ.ID02, several potentially regulatory sequences have been putatively identified (from the 5' end in the direction of the 3' end, the positions of which are indicated in base pairs (bp) with respect to the transcription initiation start point +1, as illustrated in Figure IV):

- a 104 bp sequence, reported as being responsible for conferring strong expression in green tissues and stretching from position -220 bp to position -116 bp, designated as GT in Figure IV;

- an "as-1 like" element of 16 bp in length, having a certain similarity to the activating sequence 1 (as-1) present in the CaMV 35S promoter, and stretching from position -219 bp to position -203 bp ;

- 7 "GTAA" elements specific for the expression in green tissues and located at positions -437, -216, -144, -130, -116 bp, +16 and +63 bp ;

- 7 "endosperm like" boxes having a certain similarity to the boxes responsible for specific expression in the endosperm of cereal plants, and located at positions -196, -145, -136, -130, -122, +14 and +31 bp ;

- a "TATA" box, at position -33 bp;

- the initiation start point of transcription +1 (position +1);

- a 5' untranslated region (UTR) stretching from position +1 to position 71 bp.

2.1. Construction of MPr1116.

The 104 bp sequence of CsVMV stretching from position -221 bp to position -116 bp (as referenced by the start point of initiation of transcription +1), bears 4 "endosperm like" boxes, four "GTAA" elements specific to expression in green tissues, and an as-1 type element, and is responsible for the expression in vascular tissues. The 76 bp region of CoYMV (stretching from position -160 bp to position -84 bp as identified in the sequence listing under

the number SEQ.ID01), is responsible for the expression in
vascular tissues.

The promoter MPr1116, as schematically illustrated in Figure IV,
was created by fusing the sequence of 104 bp of the promoter from
5 the intergenic region of the CsVMV genome, identified in the
sequence listing under SEQ.ID02, to the CoYMV sequence deleted of
its 76 bp region, using the lb-PCR technique.

This technique combines a ligation chain reaction, termed LCR
(Barany, 1991) that produces a single stranded continuous DNA
10 starting from "directional" oligodesoxynucleotide building blocks,
with a PCR reaction leading to the production of double stranded
DNA.

The single stranded continuous DNA was formed starting from the
following "directional" desoxynucleotides :

15 - S1 = 5'
CATGCTGCAGACTAGTATCCGCCGTCATCAATGACATCATCACAGTACTGAGGAGATGAATAGCT
3' (SEQ.ID08)

- S2 = 5'
AGCCATGACACTCTGTGCGAATATTGAAGACGTAAGCACTGACGACAACAATGAAAAGAA 3'
20 (SEQ.ID09)

- S3 = 5'
GAAGATAAGGTCGGTGATTGTGAAAGAGACATAGAGGACACATGTAAGGTGGAAAATGTAAG 3'
(SEQ.ID10)

- S4 = 5'
25 GGCGGAAAGTAACCTTATGCATTTGTAACTTGGTTACCCGGTATGCCGGTTCCCAAGCTTTAT 3'
(SEQ.ID11)

- S5 = 5'
TTCCTTATTTAAGCACTTGTGTAGTAGCTTAGAAAACCAACACAACAACCTAGAGGATCCCCG 3'
(SEQ.ID12)

30 One hundred picomoles of desoxynucleotides S1, S2 and S3 were
phosphorylated in the 5' region by the action of 15 units of
kinase (Amersham) in the presence of 5 µl 10X kinase buffer
(Amersham) and 500 picomoles of ATP (Sigma), for 30 minutes at
37°C. The phosphorylated oligodesoxynucleotides were purified by
35 extraction with a volume of phenol, then a volume of phenol :
chloroform : isoamyl alcohol (25:24:1 v/v/v) and finally a volume
of chloroform : isoamyl alcohol (24:1 v/v), before being

precipitated with of 1/10 volume 3M sodium acetate pH 4.8 et 2.5
volumes absolute ethanol at -80°C for 20 min then centrifuged at
16060 g for 30 min. The precipitated oligodesoxynucleotides were
washed in 70% ethanol, dried, then resuspended in water at a
concentration of 10 pmol/µl.

In order to link the "directional" oligodesoxynucleotides, the
following "guide" oligodesoxynucleotides were used :

- G1 = 5' GACTCCTCTACTTATCGATCGGTACTGTGAGACA 3' (SEQ.ID15)
- G2 = 5' GCTGTTGTTACTTTTCTTCTTCTATTCCAGCCA 3' (SEQ.ID16)
- G3 = 5' ATTCCACCTTTTACATTCCCGCCTTTCATTG 3' (SEQ.ID17)
- G4 = 5' CAAGGGTTCGAAATAAAGGAATAAATTCGTGA 3' (SEQ.ID18)

In order to carry out the LCR reaction, 10 pmol of the
phosphorylated "directional" desoxynucleotides S1, S2, S3, S4, and
S5 were ligated in the presence of 10 pmol of the "guide"
desoxynucleotides G1, G2, G3 and G4, 5 µl 10X Taq DNA ligase
buffer (New England Biolabs) and 40 units Taq DNA ligase (New
England Biolabs). The ligation reaction was carried out in a
GeneAmp PCR System 9700 thermocycle (Perkin Elmer, Norwalk, USA).
It consisted of a cycle of 1 min at 94°C, and 8 identical cycles
each consisting of the succession of the following steps : 1 min
at 65°C, 1 min at 57°C, 1 min at 52°C, 1 min at 48°C, 1 min at
43°C, and finally 10 min at 37°C. Then the ligation reaction
mixture was purified on a QIAquick column according to the
supplier's recommendations.

Finally, PCR amplification of the single stranded DNA obtained was
effected in a GeneAmp PCR System 9700 thermocycle in the presence
of 100 pmol of each of the oligodesoxynucleotide probes 5'
CATGCTGCAGACTAGTATCC 3' and 5' CGGGGATCCTCTAGGTTGT 3', 50 nmol of
each of the dNTP, 10 µl of Vent 10X DNA polymerase buffer (New
England Biolabs), and 2 units of DNA Vent polymerase (New England
Biolabs). The DNA was denatured for 5 min at 94°C, subjected to 25
cycles each consisting of a 30 sec denaturing step at 95°C, of a
30 sec hybridisation step at 55°C, and a 45 sec elongation step at
72°C, and then elongation at 72°C was continued for 5 min.

The DNA fragments from the reaction mixture were digested for 45
min at 37°C with 20 units of BamHI, then for 1 h at 37°C with 20
units of PstI, and finally purified on a QIAquick column. They

were inserted into the plasmid pGEM3Z-*petE* prom (described at section 1.2.) digested for 1 h at 37°C with BamHI then for 1 h at 37°C with PstI, subjected to 0.8% agarose gel electrophoresis, purified on a QIAquick affinity column, dephosphorylated for 1 h
5 at 37°C in the presence of 12 µl of 10X buffer 3 (New England Biolabs) and 5000 units of calf intestine alkaline phosphatase (CIP, New England Biolabs), and finally purified on a QIAquick affinity column. To carry out ligation, 25 ng of the plasmid treated as described above was contacted with 100 ng of the DNA
10 fragments obtained from the PCR reactions, in the presence of 1.2 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs) for 1 night at 18°C. Previously prepared Escherichia coli DH5α bacteria were transformed with half of the ligation reaction mixture. The DNA of
15 the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method, and analysed by enzymatic digestion and gene amplification using the desoxynucleotides 5' CATGCTGCAGACTAGTATCC 3' selected on the promoter and 5' TTGATTTCACGGGTTGGG 3' selected
20 on the *uidA* sequence. Two resulting plasmids pMRT1116 and pMRT1117 were sequenced. The plasmid pMRT1116 contains the promoter MPr1116 (SEQ.ID03), whereas the plasmid pMRT1117 bears the promoter MPr1117 (SEQ.ID04) which differs from MPr1116 by a duplication, in the 5' region of the chimeric promoter, of the "as-1 like" box and
25 its immediate environment, the length of which is 33 bp, as well as the deletion of 3 bp at position -140, -25 and -24, and the replacement of a cytosine with a thymine at position -54 bp (as illustrated by Figure IV).

2.2. Construction of the promoter MPr1146 :
30 The promoter MPr1146 (Fig. IV) was obtained by inserting the 58 bp sequence corresponding to a duplication of the as-2 element (Lam et Chua, 1989) and of the as-1 element (Lam *et al.,* 1989) originating from the RNA 35S CaMV promoter at the restriction sites NheI and DraIII of MPr1116.
35 In order to achieve this, the plasmid pMRT1116 was digested for 1 h at 37°C with 25 units of NheI, then for 1 h at 37°C with 4 units of DraIII. The plasmid thus digested was isolated on 0.8% agarose

gel, purified on a QIAquick affinity column and dephosphorylated
for 1 h at 37°C in the presence of 12 μl of 10X buffer 3 (New
England Biolabs) and 5000 units of calf intestine alkaline
phosphatase (CIP, New England Biolabs), and finally purified once
5 again on a QIAquick affinity column.

The fragment SpeI/DraIII of 70 bp containing the two as-2 elements
and the as-1 element was obtained from the plasmid pMRT1111.
This latter plasmid, which contains a 58 bp sequence
corresponding to a duplication of the as-2 element (Lam et Chua,
10 1989) and an as-1 element (Lam *et al.*, 1989) originating from the
35S RNA CaMV promoter upstream of the minimal pea plastocyanin
promoter modified by the addition of a "G" box, was obtained by
lb-PCR in the following manner. The single stranded continuous DNA
was generated using the following "directional" desoxynucleotides
15 :

– S1 = 5'
TTCCCTTCAAACACATACAAATTCAGTAGAGAAGAAACTCATTACTCTTGAGAAACCTAGAGGATC
CCCG 3' (SEQ.ID08)

20 – S2 = 5'
CACAAAAACCCAATCCACATCTTTATCATCCATTCTATAAAAAATCACCTTCTGTGTGTCTCTCTT
TCGA 3' (SEQ.ID09)

– S5 = 5'
CTGTGGCACATCTACATTATCTAAATCTAAGCCACGTCGGAGGATAACATATTCTTCCACACATCT
25 TAGCCA 3' (SEQ.ID12)

– S7 = 5'
CATGCTGCAGACTAGTGATTGATGTGATATCAAGATTGATGTGATATCTCCACTGACGTAAGGGAT
GACGCATGCCACT 3' (SEQ.ID14)

One hundred picomoles (100 pmol) of the desoxynucleotides S1, S2
30 and S5 were 5' phosphorylated with 15 units of kinase (Amersham)
in the presence of 5 μl 10X kinase buffer (Amersham) and 500 pmol
of ATP (Sigma), for 30 min at 37°C. The phosphorylated
oligodesoxynucleotides were purified by extraction with a volume
of phenol, then a volume of phenol : chloroform : isoamyl alcohol
35 (25:24:1 v/v/v) and finally a volume of chloroform : isoamyl
alcohol (24:1 v/v), before being precipitated with 1/10 volume 3M
sodium acetate pH 4.8 and 2.5 volumes absolute ethanol at −80°C

for 20 min then centrifuged at 16060 g for 30 min. The
precipitated oligodesoxynucleotides were washed in 70% ethanol,
dried, then resuspended in water at a concentration of 10 pmol/µl.
In order to link the "directional" oligodesoxynucleotides, the
5 following "guide" oligodesoxynucleotides were used :
  - G1= 5' TGTGTTTGAAGGGAATCGAAAGAGAGACACA 3' (SEQ.ID15)
  - G2= 5' GATTGGGTTTTTGTGTGGCTAAGATGTGTG 3' (SEQ.ID16)
  - G4= 5' TGTAGATGTGCCACAGAGTGGCATGCGT 3' (SEQ.ID18)
In order to carry out the LCR reaction, 10 pmol of the
10 phosphorylated directional desoxynucleotides S1, S2, S5 and S7
were ligated in the presence of 10 pmol of the "guide"
desoxynucleotides G1, G2 and G4, 5 µl Taq 10X DNA ligase buffer
(New England Biolabs) and 40 units Taq DNA ligase (New England
Biolabs). The ligation reaction was carried out in a GeneAmp PCR
15 System 9700 thermocycle (Perkin Elmer, Norwalk, USA). It consists
of a cycle of 1 min at 94°C, and 8 identical cycles each
consisting of the succession of the following steps : 1 min at
65°C, 1 min at 57°C, 1 min at 52°C, 1 min at 48°C, 1 min at 43°C,
and finally 10 min at 37°C. Then, the ligation reaction mixture
20 was purified on a QIAquick affinity column according to the
supplier's recommendations.
Finally, PCR amplification of the single stranded continuous DNA
obtained was carried out in a GeneAmp PCR System 9700 thermocycle
in the presence of 100 pmol of each of the following
25 oligodesoxynucleotide probes 5' CATGCTGCAGACTAGTGGATT 3', and 5'
CGGGGATCCTCTAGGTTTCT 3', 50 nmol of each of the dNTP, 10 µl of
Vent 10X DNA polymerase buffer (New England Biolabs), and 2 units
of Vent DNA polymerase (New England Biolabs). The DNA was
denatured for 5 min at 94°C, subjected to 25 cycles each
30 consisting of a 30 sec denaturing step at 95°C, a 30 sec
hybridisation step at 56°C, and a 1 min elongation step at 72°C,
then further elongation at 72°C for 5 min.
The DNA fragments of the reaction mixture were digested for 45 min
at 37°C with 20 units of BamHI, then for 1 h at 37°C with 20 units
35 of PstI, and finally purified on a QIAquick column. They were
inserted into the plasmid pGEM3Z-petE prom digested for 1 h at
37°C with BamHI, then for 1 h at 37°C with PstI, subjected to 0.8%

agarose gel electrophoresis, purified on a QIAquick affinity
column, dephosphorylated for 1 h at 37°C in the presence of 12 μl
of 10X buffer 3 (New England Biolabs) and 5000 units of calf
intestine alkaline phosphatase (CIP, New England Biolabs), and
5 finally purified on a QIAquick affinity column. In order to carry
out the ligation, 25 ng of plasmid treated as described above were
contacted with 100 ng of the DNA fragments obtained by PCR, in
the presence of 1,2 μl of T4 10X DNA ligase buffer (New England
Biolabs) and 400 units T4 DNA ligase (New England Biolabs) for 1
10 night at 18°C. Previously prepared viable and competent
Escherichia coli DH5α bacteria were transformed with half of the
ligation reaction mixture. The DNA of the obtained clones,
selected on LB media supplemented with ampicillin (50 mg/l), was
extracted according to the alkaline lysis method and was analysed
15 by enzymatic digestion. The promoter sequence MPr1111 of one of
these clones was verified by sequencing.
The 70 bp fragment containing the two as-2 elements and the as-1
element was obtained by digesting 25 μg of plasmid pMRT1111 with
40 units of SpeI for 1 h at 37°C, then with 4 units of DraIII for
20 1 h at 37°C. The fragment was isolated by electrophoresis on
Nu-Sieve 3% gel agarose (FMC, Rockland, USA) and finally purified
on a QIAquick affinity column.
The ligation was carried out with 30 ng of dephosphorylated
plasmid vector pMRT1116 NheI/DraIII and 50 ng of the 70 bp
25 fragment for 15 h at 18°C in a reaction volume of 20 μl in the
presence of 2.0 μl T4 10X DNA ligase buffer (New England Biolabs)
and 800 units T4 DNA ligase (New England Biolabs). Previously
prepared viable and competent Escherichia coli DH5α bacteria were
transformed with half of the ligation reaction mixture. The
30 plasmid DNA of the obtained clones, selected on LB media
supplemented with ampicillin (50 mg/l), was extracted according
to alkaline lysis method, and analysed by enzymatic digestion and
gene amplification using the desoxynucleotides 5'
CATGCTGCAGACTAGTATCC 3' selected on the promoter and 5'
35 TTGATTTCACGGGTTGGG 3' selected on the uidA sequence. The promoter
sequence MPr1146 (SEQ.ID05) of one of these clones was verified by

sequencing.

2.3. Construction of the promoter MPr1147 :

The promoter MPr1147 (Fig. IV) was obtained by inserting a
sequence of 44 bp from the RNA 35S CaMV promoter containing the
elements as-2 and as-1 (Lam, 1989 ; Lam et al., 1989) and
restriction sites adjacent to sites NheI and DraIII of MPr1117
(SEQ.ID04).

In order to achieve this, the plasmid pMRT1117 was digested with
25 units of NheI for 1 h at 37°C then purified on a QIAquick
affinity column. The ends generated were blunted by the action of
Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the
supplier's recommendations, then the plasmid obtained was
digested for 1 h at 37°C with 4 units of DraIII. The thus modified
plasmid was isolated on 0.8% agarose gel, purified on a QIAquick
affinity column and dephosphorylated for 1 h at 37°C in the
presence of 12 µl of 10X buffer 3 (New England Biolabs) and 5000
units of calf intestine alkaline phosphatase (CIP, New England
Biolabs), and finally purified again on a QIAquick affinity
column.

The 54 bp fragment PstI/DraIII containing the 44 bp of the as-2
and as-1 elements of the CaMV 35S was obtained from plasmid
pMRT1110. The plasmid pMRT1110, which contains a sequence of 44
bp corresponding to the as-2 element (Lam et Chua, 1989) and the
as-1 element (Lam et al., 1989) of the 35S RNA CaMV promoter
upstream of the minimal pea plastocyanine promoter modified by the
addition of a "G" box, was obtained by 1b-PCR in the following
manner. The single stranded continuous DNA was formed using the
"guide" desoxynucleotides :

- S1 = 5'

TTCCCTTCAAACACATACAAATTCAGTAGAGAAGAAACTCATTACTCTTGAGAAACCTAGAGGATC
CCCG 3' (SEQ.ID08)

- S2 = 5'

CACAAAAACCCAATCCACATCTTTATCATCCATTCTATAAAAAATCACCTTCTGTGTGTCTCTCTT
TCGA 3' (SEQ.ID09)

- S5 = 5'

CTGTGGCACATCTACATTATCTAAATCTAAGCCACGTCGGAGGATAACATATTCTTCCACACATCT
TAGCCA 3' (SEQ.ID12)

- S6 =
5'CATGCTGCAGACTAGTGGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCATGCCACT
3' (SEQ.ID13)

One hundred picomoles (100 pmol) of the desoxynucleotides S1, S2
5   and S5 were phosphorylated in 5' by 15 units of kinase (Amersham)
in the presence of 5 µl 10X kinase buffer (Amersham) and 500 pmol
ATP (Sigma), for 30 min at 37°C. The phosphorylated
oligodesoxynucleotides were purified by extraction extraction with
a volume of phenol, then a volume of phenol : chloroform : isoamyl
10 alcohol (25:24:1 v/v/v) and finally a volume of chloroform :
isoamyl alcohol (24:1 v/v), before being precipitated with 1/10
volume 3M sodium acetate pH 4.8 et 2.5 volumes absolute ethanol at
-80°C for 20 min then centrifuged at 16060 g for 30 min. The
precipitated oligodesoxynucleotides were washed in 70% ethanol,
15 dried, then resuspended in water at a concentration of 10 pmol/µl.
In order to link the "directional" oligodesoxynucleotides , the
following "guide" oligodesoxynucleotides were used :
- G1= 5' TGTGTTTGAAGGGAATCGAAAGAGAGACACA 3' (SEQ.ID15)
- G2= 5' GATTGGGTTTTTGTGTGGCTAAGATGTGTG 3' (SEQ.ID16)
20 - G4= 5' TGTAGATGTGCCACAGAGTGGCATGCGT 3' (SEQ.ID18)
In order to carry out the LCR reaction, 10 pmol of the
phosphorylated "directional" desoxynucleotides S1, S2, S5 and S6
were ligated in the presence of 10 pmol of the "guide"
desoxynucleotides G1, G2 and G4, 5 µl Taq 10X DNA ligase buffer
25 and 40 units Taq DNA ligase (New England Biolabs). The ligation
reaction was carried out in a GeneAmp PCR System 9700 thermocycle.
It consists of a cycle of 1 min at 94°C, and 8 identical cycles
each consisting of the succession of the following steps : 1 min
at 65°C, 1 min at 57°C, 1 min at 52°C, 1 min at 48°C, 1 min at
30 43°C, and finally 10 min at 37°C. Then, the ligation reaction
mixture was purified on a QIAquick column according to the
supplier's recommendations.
Finally, PCR amplification of the single stranded DNA obtained
was carried out in a thermocycleur GeneAmp PCR System 9700 in the
35 presence of 100 pmol of each of the oligodesoxynucleotide probes
5' CATGCTGCAGACTAGTGGATT 3', and 5' CGGGGATCCTCTAGGTTTCT 3', of 50
nmol of each of the dNTP, of 10 µl of 10X Vent DNA polymérase

buffer (New England Biolabs), and 2 units Vent DNA polymerase (New England Biolabs). The DNA was denatured for 5 min at 94°C, subjected to 25 cycles each consisting of a 30 sec denaturing step at 95°C, a 30 sec hybridisation step at 56°C, and of 1 min
5   elongation at 72°C, then further elongation at 72°C for 5 min. The DNA fragments of the reaction mixture were digested for 45 min at 37°C with 20 units of BamHI then for 1 h at 37°C with 20 units of PstI, and finally purified on a QIAquick column. They were inserted in the plasmid pGEM3Z-petE prom digested for 1 h at 37°C
10 with BamHI then for 1 h at 37°C with PstI, subjected to 0.8% agarose gel electrophoresis, purified on a QIAquick affinity column, dephosphorylated for 1 h at 37°C in the presence of 12 µl of 10X buffer 3 (New England Biolabs) and 5000 units of calf intestine alkaline phosphatase (CIP, New England Biolabs), and
15 finally purified on a QIAquick affinity column. To carry out the ligation, 25 ng of plasmid treated as described above were contacted with 100 ng of the DNA fragments obtained by PCR, in the presence of 1.2 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units T4 DNA ligase (New England Biolabs) for 1
20 night at 18°C. Previously prepared viable and competent Escherichia coli DH5α bacteria were transformed with half of the ligation reaction mixture. The DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to alkaline lysis method and analysed by
25 enzymatic digestion. The promoter sequence MPr1110 borne by this plasmid pMRT1110 was verified by sequencing.
The 54 bp fragment containing the sequences as-2 and as-1 originating from the CaMV promoter was obtained by digesting 25 µg of the plasmid pMRT1110 for 1 h at 37°C with 80 units of PstI,
30 then the generated ends were blunted by the action of Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the supplier's recommendations. The plasmid thus modified was digested for 1 h at 37°C with 4 units of DraIII, and the 54 bp fragment was isolated by electrophoresis on 3% Nu-Sieve agarose gel (FMC,
35 Rockland, USA) and finally purified on a QIAquick affinity column. The ligation was carried out with 30 ng of vector pMRT1117 prepared as described previously and 50 ng of the 54 bp fragment

for 15 h at 18°C in a reaction volume of 20 µl in the presence of 2.0 µl of T4 10X DNA ligase buffer (New England Biolabs) and 800 units T4 DNA ligase (New England Biolabs). Previously prepared viable and competent Escherichia coli DH5α bacteria were

5 transformed with half of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method , and analysed by enzymatic digestion and gene amplification using the desoxynucleotides 5'

10 CATGCTGCAGACTAGTATCC 3' selected from the promoter and 5' TTGATTTCACGGGTTGGG 3' selected from the uidA sequence. The promoter sequence MPr1147 (SEQ.ID06) of one of these clones was verified by sequencing.

2.4. Construction of the promoter MPr1154 :

15 The promoter MPr1154 (Fig. IV) was obtained by deleting a 44 bp sequence containing the duplicated as-1-like element from CoYMV present in the promoter MPr1147 (SEQ.ID04).

In order to achieve this, the plasmid pMRT1147 was digested with 20 units of SpeI for 1 h at 37°C, isolated on 0.8% gel agarose,

20 purified on a QIAquick affinity column and religated for 15 h at 18°C in a reaction volume of 10 µl in the presence of 1 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units T4 DNA ligase (New England Biolabs). Previously prepared viable and competent Escherichia coli DH5α bacteria were transformed with

25 half of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method, and analysed by enzymatic digestion and gene amplification using the desoxynucleotides 5' ATTTAGGTGACACTATAG 3'

30 selected from the plasmid and 5' TTGATTTCACGGGTTGGG 3' selected from the uidA sequence. The promoter sequence MPr1154 (SEQ.ID07) of one of these clones was verified by sequencing .

2.5. Construction of the promoters MPr1162, MPr1163, MPr1164, MPr1165 :

35 The promoters MPr1162, MPr1163, MPr1164, and MPr1165 (Figure VII) were obtained by inserting one or multiple copies of the 70 bp

sequence corresponding to a duplication of the as-2 box (Lam and
Chua, 1989) and the as-1 box (Lam et al., 1989) of the 35S RNA
CaMV promoter, bearing restriction sites on either side, into the
site BstEII from MPr1116 (SEQ.ID03), in the 5'>3' orientation or
5   inverted, that is to say, in the 3'>5' orientation.

In order to do this, the plasmid pMRT1116 was digested with 4
units of BstEII for 1 h at 37°C then purified on a QIAquick
affinity column. The generated ends were blunted by the action of
Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the
10 recommendations of the supplier, then dephosphorylated for 1 h at
37°C in the presence of 12 µl of 10X "buffer 3" (New England
Biolabs) and 25 units of calf intestine alkaline phosphatase (CIP,
New England Biolabs). Finally, the thus obtained plasmid was
purified again on a QIAquick affinity column.

15 The 70 bp SpeI/DraIII fragment containing the two "as-2" boxes and
the "as-1" box was obtained from plasmid pMRT1111.

The plasmid pMRT1111, which contains a 58 bp sequence
corresponding to a duplication of the as-2 box (Lam et Chua, 1989)
and the as-1 box (Lam et al., 1989) from the 35S RNA CaMV promoter
20 placed upstream of the minimal pea plastocyanine promoter modified
by the addition of a "G" box, was obtained by lb-PCR in the
following way. The single stranded DNA was produced with the help
of the following directional oligodesoxynucleotides :

– S1 = 5'
25 TTCCCTTCAAACACATACAAATTCAGTAGAGAAGAAACTCATTACTCTTGAGAAACCTAGAGGATC
CCCG 3' (SEQ.ID08)

– S2 = 5'
CACAAAAACCCAATCCACATCTTTATCATCCATTCTATAAAAAATCACCTTCTGTGTGTCTCTCTT
TCGA 3' (SEQ.ID09)

30 – S5 = 5'
CTGTGGCACATCTACATTATCTAAATCTAAGCCACGTCGGAGGATAACATATTCTTCCACACATCT
TAGCCA 3' (SEQ.ID12)

– S7 = 5'
CATGCTGCAGACTAGTGATTGATGTGATATCAAGATTGATGTGATATCTCCACTGACGTAAGGGAT
35 GACGCATGCCACT 3' (SEQ.ID14)

One hundred picomoles (100 pmol) of the S1, S2 et S5
oligodesoxynucleotides were 5' phosphorylated with 15 units of

kinase (Amersham) in the presence of 5 μl 10X kinase buffer
(Amersham) and 500 pmol ATP (Sigma), for 30 min at 37°C. The
phosphorylated desoxynucleotides were purified by extraction with
a volume of phenol, then a volume of phenol : chloroform : isoamyl
5   alcohol (25:24:1 v/v/v) and finally a volume of chloroform :
isoamyl alcohol (24:1 v/v), before being precipitated by 1/10
volume 3M sodium acetate pH 4.8 and 2.5 volumes absolute ethanol
at −80°C for 20 min then centrifuged at 16060 g for 30 min. The
precipitated oligodesoxynucleotides were washed in 70% ethanol,
10   dried, then resuspended in water at a concentration of 10 pmol/ml.
In order to link the directional oligodesoxynucleotides, the
following guide oligodesoxynucleotides were used :
− G1= 5′ TGTGTTTGAAGGGAATCGAAAGAGAGACACA 3′ (SEQ.ID15)
− G2= 5′ GATTGGGTTTTTGTGTGGCTAAGATGTGTG 3′ (SEQ.ID16)
15   − G4= 5′ TGTAGATGTGCCACAGAGTGGCATGCGT 3′ (SEQ.ID18)
In order to carry out the LCR reaction, 10 pmol of the
phosphorylated oligodesoxynucleotides S1, S2, S5 and S7 were
ligated in the presence of 10 pmol of the guide
oligodesoxynucleotides G1, G2 and G4, 5 μl of Taq 10X DNA ligase
20   buffer (New England Biolabs) and 40 units Taq DNA ligase (New
England Biolabs). The ligation reaction was carried out in a
"GeneAmp PCR System 9700" thermocycle (Perkin Elmer, Norwalk,
USA). It consisted of a cycle of 1 min at 94°C, and 8 identical
cycles each consisting of the succession of the following steps :
25   1 min at 65°C, 1 min at 57°C, 1 min at 52°C, 1 min at 48°C, 1 min
at 43°C, and finally 10 min at 37°C. Then, the ligation reaction
mixture was purified on a QIAquick column according to the
suppliers's recommendations.
Finally, PCR amplification of the the single stranded DNA obtained
30   was carried out in a "GeneAmp PCR System 9700" thermocycle in the
presence of 100 pmol of each of the oligodesoxynucleotide probes
5′ CATGCTGCAGACTAGTGGATT 3′, and 5′ CGGGGATCCTCTAGGTTTCT 3′, 50
nmol of each dNTP, 10 μl of Vent 10X DNA polymerase buffer (New
England Biolabs), and 2 units Vent DNA polymerase (New England
35   Biolabs). The DNA was denatured for 5 min at 94°C, subjected to 25
cycles each consisting of a 30 sec denaturing step at 95°C, of a
30 sec hybridisation step at 56°C, and of 1 min elongation at

72°C, then further elongation at 72°C for 5 min.

The DNA fragments from the reaction mixture were digested for 45 min at 37°C by 20 units of BamHI, then for 1 h at 37°C by 20 units of PstI, and finally purified on a QIAquick column. They were inserted into the plasmid pGEM3Z-*petE* prom previously digested for 1 h at 37°C by BamHI, then for 1 h at 37°C by PstI, subjected to electrophoresis on 0.8% agarose gel, purified on a QIAquick affinity column, dephosphorylated for 1 h at 37°C in the presence of 12 µl of 10X "buffer 3" (New England Biolabs) and 25 units of calf intestine alkaline phosphatase (CIP, New England Biolabs), and finally purified on a Qiaquick affinity column. In order to carry out ligation, 25 ng of the thus treated plasmid was contacted with 100 ng of the DNA fragments obtained by PCR, in the presence of 1.2 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs) for 1 night at 18°C. Previously prepared viable and competent *Escherichia coli* DH5α bacteria were transformed half of the ligation reaction mixture. The DNA of the obtained clones, selected on LB media supplemented with ampicillin (50 mg/l), was extracted according to the alkaline lysis method and analysed y enzymatic digestion. The promoter sequence MPr1111 of one of the obtained clones was verified by sequencing.

The 70 bp fragment containing the two "as-2" boxes and the "as-1" box were obtained by digestin 25 mg of plasmid pMRT1111 with 40 units of SpeI for 1 h at 37°C, then with 4 units of DraIII for 1 h at 37°C. The fragment was isolated by electrophoresis on Nu-Sieve 3 % agarose gel (FMC, Rockland, USA) and finally purified on a QIAquick affinity column. The ends of this fragment were blunted by the action of Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the recommandations of the supplier, then purified again on a QIAquick affinity column.

The ligation was carried out with 30 ng of pMRT1116 vector prepared as described above and 50 ng of 70 bp fragment for 15 h at 18°C in a reaction mixture of 20 µl in the presence of 2.0 µl of 10X T4 DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs). Previously prepared viable and competent *Escherichia coli* DH5α bacteria, were transformed

with a third of the ligation reaction mixture. The plasmid DNA of
the obtained clones, selected on LB media supplemented with
ampicillin (50 mg/l), was extracted according to the alkaline
lysis method, and analysed by enzymatic digestion and by gene
5 amplification with the help of the universal SP6
oligodesoxynucleotide (5' TAAATCCACTGTGATATCTTATG 3') located in
the 5' region of the promoter and the oligodesoxynucleotide
5' TTGATTTCACGGGTTGGG 3' selected on the *uidA* sequence. This
cloning strategy enabled the production of 70 bp fragment inserts
10 into the vector in the 5'>3' orientation (the sequences
as-2/as-2/as-1 are cloned in the same orientation as in their
native promoter), in 3'>5' antisens orientation (an oriention
inverse to that present in the CaMV 35S promoter) and in one or
more copies. The following synthetic and chimeric promoters could
15 be obtained using this strategy : MPr1162 (SEQ.ID19), which
corresponds to the insertion of a single 70 bp sequence in normal
5'>3' orientation, MPr1163 (SEQ.ID20), which corresponds to the
insertion of two 70 bp sequences in the normal 5'>3' orientation,
MPr1164 (SEQ.ID21), which corresponds to the insertion of a single
20 70 bp sequence in inverse or antisens orientation, and MPr1165
(SEQ.ID22), which corresponds to the insertion of four 70 bp
sequences in normal 5'>3' orientation. Each one of these clones
was verified by sequencing.

25 **2.6. Construction of promoters MPr1167, MPr1168, and MPr1169 :**
The promoters MPr1167, MPr1168 and MPr1169 (Fig. VII) are derived
from MPr1147 (SEQ.ID06). They were obtained by inserting, into the
BstEII site of MPr1147, one or more normally 5'>3' oriented (i.e.
cloned in the same orientation as the native orientation of the
30 sequences in the CaMV promoter) 70 bp sequences containing the 58
bp sequence coresponding to a duplication of the as-2 box (Lam et
Chua, 1989) and the as-1 box (Lam *et al.,* 1989) of the 35S RNA
CaMV promoter.
In order to do this, the plasmid pMRT1147 was digested with 4
35 units of BstEII for 1 h at 37°C then purified on a Qiaquick
affinity column. The generated ends were blunted by the action of
Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the

recommandations of the supplier, then dephosphorylated for 1 h at
37°C in the presence of 12 µl of 10X "buffer 3" (New England
Biolabs) and 25 units of calf instestine alkaline phosphatase
(CIP, New England Biolabs). Finally the plasmid thus obtained was
5 purified again on a Qiaquick affinity column.
The 70 bp SpeI/DraIII fragment containing the two "as-2" boxes and
the "as-1" box was obtained from plasmid pMRT1111, the production
of which was described previously. The ends of this fragment were
blunted as described above.
10 The ligation was carried out with 30 ng of vector pMRT1147
prepared as described previously and 50 ng of the 70 bp fragment
for 15 h at 18°C in a reaction mixture of 20 µl in the presence
of 2.0 µl 10X T4 DNA ligase buffer (New England Biolabs) and 400
units T4 DNA ligase (New England Biolabs). Previously prepared
15 viable and competent *Escherichia coli* DH5α bactéria were
transformed by reacting with a third of the ligation reaction
mixture. The plasmid DNA of the obtained clones, selected on LB
media supplemented with ampicillin (50 mg/l), was extracted
according to the alkaline lysis method, and analysed by enzymatic
20 digestion and by gene amplification using the universal SP6
oligodesoxynucleotide (5' TAAATCCACTGTGATATCTTATG 3') located in
the 5' region of the promoter and the oligodesoxynucleotide
5' TTGATTTCACGGGTTGGG 3' selected from the *uidA* sequence. The
following synthetic promoters could be prepared by the preceding
25 method : MPr1167 (SEQ.ID23), which corresponds to the insertion of
three 70 bp sequences in normal 5'>3' orientation, MPr1168
(SEQ.ID24), which corresponds to the insertion of two 70 bp
sequences in normal 5'>3' orientation and MPr1169 (SEQ.ID25),
which corresponds to the insertion of a single 70 bp sequence in
30 normal 5'>3' orientation. Each of these clones was verified by
sequencing.

**2.7. Production of binary vector pMRT1218 containing promoter
MPr1218 :**

35 The reference promoter for expression of the GUS reporter gene in
corn seed endosperm is the promoter of gene coding for a storage
protein in corn seed of 28 kDa belonging to the family of

gamma–zeins. This construct has been described in French patent
application number FR9912373, filed on September 30th, 1999, in the
name of the applicants, and is incorporated herein for the
purposes of the specific description of said reference promoter

5 and vector. The 1.7 kb γ-zein whole length promoter sequence
(Prγ-zein) contained in the plasmid p63 described by Reina et al.
(1990) was placed upstream of the uidA-IV2/term-nos sequence in a
vector designated pMRT1126. The vector pMRT1126 is described in
full in the abovementioned French patent application, and its

10 description is incorporated herein for the purposes of the
description of this vector. The promoter Mpr1126 in vector
pMRT1126 derives from the HMWG-Dx5 (High Molecular Weight
Glutenin) promoter by deletion of the sequence situated upstream
of nucleotide –142, which sequence comprises two "prolamine like"

15 boxes, two "GATA" boxes, a "G" box and and an activator element.
The promoter fragment was amplified by PCR and isolated.
The 1.7 kb γ-zein promoter was obtained by digesting 15 µg of
plasmid p63 with restriction enzymes HindIII and BamHI for 1 h at
37°C. The thus liberated 1.7 kb Prγ-zein fragment was isolated on

20 0.8% agarose gel using a « Concert Rapid Gel Extraction System »
kit.
In parallel, 10 µg of plasmid pMRT1126 were digested by the
restriction enzymes HindIII and BamHI for 1 h at 37°C. The vector
fragment was then isolated on 0.8% agarose gel using a « Concert

25 Rapid Gel Extraction System » kit and dephosphorylated with 40
units of calf intestine alkaline phosphatase (New England Biolabs)
in the presence of 10X "buffer 3" at 37°C for 1 h.
The ligation reaction was carried out with 50 ng of γ-zein promoter
fragment and 100 ng of plasmid pMRT1126, in a reaction volume of

30 10 µl, in the presence of tampon T4 10X DNA ligase and 400 units
of T4 DNA ligase (New England Biolabs), in a « GeneAmp PCR System
9700 » thermocycle as described above. Previously prepared viable
and competent Escherichia coli DH5α bacteria were transformed with
all of the ligation reaction mixture. The plasmid DNA of the

35 obtained clones, selected on LB media supplemented with ampicillin
(50 mg/l), was extracted according to the alkaline lysis method

and analysed by enzymatic digestion. The resulting plasmid was designated pMRT1218.

3. Construction of binary plasmids containing the promoters.

Three types of binary vector were used for cloning the various expression cassettes. The vector pGA492 was used to prepare the cassettes containing MPr1116, MPr1146, MPr1147 and MPr1092 to create the expression cassettes or binary vectors pMRT1152, pMRT1171, pMRT1172 and pGA492MPr1092 respectively. The vector pGA492 was prepared as follows. A 25 µg amount of plasmid pGA492 (An, 1986) was digested with 80 units of HindIII for 1 h at 37°C then purified on a QIAquick affinity column. The 5' protruding ends of this plasmid were blunted using Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the supplier's recommendations. The thus modified plasmid was digested with 80 units of EcoRI for 1 h at 37°C, then the vector deleted of its 291 bp fragment was isolated on 0.7% agarose gel and purified on a QIAquick affinity column. A 25 µg amount of plasmid pGA492 (An, 1986) was digested with 80 units of HindIII for 1 h at 37°C then purified on a QIAquick affinity column.

The vector pMRT1118 was used to clone the cassettes under control of the promoters MPr1162, MPr1164, MPr1165, MPr1167 and MPr1092 to give the vectors pMRT1185, pMRT1186, pMRT1187, pMRT1188 and pMRT1182 respectively.

The plasmid pMRT1118 is described completely in French patent application number FR9911112, filed on September 3, 1999, also in the name of the present applicant, the specific description of which is incorporated herein by reference. The binary plasmid pMRT1118 (5971 pb) results from the introduction of a T-DNA fragment digested by AvrII enzyme into the AvrII site of another dephosphorylated plasmid also fully described in the previously mentioned prior application to same applicant, and designated pMRT1106, also specifically incorporated herein by reference.

In order to carry out the insertion, the pMRT1106 plasmid DNA (5 µg) was digested with AvrII enzyme, purified with the aid of the «QIAquick PCR Purification» kit, then dephosphorylated with 50 units of calf intestine alkaline phosphatase (New England Biolabs)

in a final reaction mixture volume of 120 µl in the presence of 12 µl 3x 10 buffer (New England Biolabs) at 37 °C for 1 hour, isolated by electrophoresis on at 0.6% agarose gel in TBE buffer, purified with a «QIAquick Gel Extraction» kit, dephosphorylated a

5 second time with the calf intestine alkaline phosphatase under the conditions mentioned above, and finally purified with a «QIAquick PCR Purification» kit and transferred to 50 µl de H_2O.

The PCR ligation reaction was carried out with 32,5 ng of digested dephosphorylated plasmid pMRT1106 and 50 ng of T-DNA fragments

10 digested in a reaction mixture volume of 10 µl in the presence of 1 µl T4 10x DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs). The ligation comprised 180 cycles each including 2 steps, the first one at 10°C for 30 seconds and the second step at 30°C for 30 seconds in a «GeneAmp

15 PCR System 9700» thermocycle.

Previously prepared viable and competent Escherichia coli DH5α bacteria, were transformed (Hanahan, 1983). The plasmid DNA of the obtained clones, selected on LB media supplemented with kanamycin (50 mg/l), was extracted according to the alkaline lysis method

20 (Birnboim et Doly, 1979) and verified by enzymatic digestion and sequencing. The resulting plasmid was designated pMRT1118.

This vector was then prepared in the following manner : a 25 µg amount of plasmid was digested with 80 units of HindIII for 1 h at 37°C then purified on a Qiaquick affinity column. The 5'

25 protruding ends of this plasmid were blunted using Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the supplier's recommendations. The thus modified plasmid was digested with 80 units of EcoRI for 1 h at 37°C, then the vector was isolated on 0.7% agaros gel and purified on a Qiaquick affinity column. This

30 was then dephosphorylated for 1 h at 37°C using 10 units of calf intestine alkaline phosphatase (New England Biolabs) according to the manufacturer's recommendations, then purified on a QIAquick affinity column.

The vector pMRT1195 was used to clone the cassettes under the

35 control of the promoters MPr1116, MPr1154, MPr1162, MPr1163, MPr1164, MPr1165, MPr1167, MPr1168, MPr1169 and MPr1092 to give the vectors pMRT1245, pMRT1246, pMRT1247, pMRT1248, pMRT1249,

pMRT1250, pMRT1251, pMRT1252, pMRT1253 and pMRT1254 respectively.
The vector pMRT1195, which is also described in the prior French
application number FR 9911112, filed on September 3, 1999, also in
the name of the present applicant, and incorporated herein by
5 reference to that particular aspect, comprises in order : an ori
RK2 region followed by an ori ColEI region, followed by nptIII and
an nptII regions and then a trfA region, subsequently followed by
a transfer DNA left border region, a nos terminator region, a bar
region (coding for phosphinotricine acetyl transferase, a protein
10 conferring herbicide resistance); a rice actin intron-1 region, a
polyadenylation transcription termination signal region, a
multiple cloning site region and then a transfer DNA right border
region. The vector pMRT1195 was prepared in the following manner.
A 20 µg amount of plasmid pMRT1195 was digested by 15 units of
15 HpaI for 1 h at 37°C then purified on a QIAquick affinity column.
The thus opened vector was dephosphorylated for 1 h at 37°C using
10 units of calf intestine alkaline phosphatase (New England
Biolabs) according to the manufacturer's recommendations, then
purified on a QIAquick affinity column.
20 **3.1. Production of pMRT1152.**
The expression cassette MPr1116 / uidA-IV2 / nos term was cloned
at the modified HindIII site of the binary plasmid pGA492.
It was obtained from the plasmid pMRT1116 digested with 80 units
of PstI for 1 h at 37°C and purified on a QIAquick affinity
25 column. The protruding 5' ends of this plasmid were blunted using
Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the
supplier's recommendations. The plasmid thus modified was digested
simultaneously with 80 units of EcoRI and 40 units of XmnI for 1 h
at 37°C, then the 2.5 kb DNA fragment corresponding to the
30 expression cassette was separated on 1% agarose gel and purified
on a QIAquick affinity column.
The ligation was carried out by mixing 100 ng of binary plasmid
pGA492 prepared as described above and 50 ng of expression
cassette for 1 night at 18°C in a reaction volume of 20 µl in
35 the presence of 2 µl of T4 10X DNA ligase buffer (New England
Biolabs) and 400 units of T4 DNA ligase (New England Biolabs).
Previously prepared viable and competent Escherichia coli DH5α

bacteria were transformed with half of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with tetracycline (12 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion as well as by gene amplification using the desoxynucleotides 5' ATATGAGACTCTAATTGGATACCGAGGGG 3' selected from the transfer DNA of the binary plasmid and 5' TTGATTTCACGGGTTGGG 3' selected from the the expression cassette in the *uidA* sequence. The resulting clone was designated pMRT1152.

3.2. Production of the binary plasmid pMRT1171.

The expression cassette MPr1146 / *uidA*-IV2 / *nos* term was cloned at the modified HindIII site of the binary plasmid pGA492 by following the same protocol as for plasmid pMRT1152, except that the expression cassette was isolated from the plasmid pMRT1146. The resulting clone was designated pMRT1171.

3.3. Production of the binary plasmid pMRT1172.

The expression cassette MPr1147 / *uidA*-IV2 / *nos* term was cloned at the modified HindIII site of the binary plasmid pGA492 by following the same protocol as for the plasmid pMRT1152, except that the expression cassette was isolated from the plasmid pMRT1147. The resulting clone was designated pMRT1172.

3.4. Production of the binary plasmid pGA492MPr1092.

The promoter fragments MPr1092 and *uidA*-IV2 / *nos* term sequence were inserted into the binary plasmid pGA492 prepared as described above. The two fragments were prepared in the following manner :

– CaMV D35S prom was isolated by digesting 10 μg of plasmid pJIT163Δ with 40 units of KpnI for 1 h at 37 °C. The ends of this linearized plasmid were blunted using 6 units of T4 DNA polymerase (New England Biolabs) for 30 min at 37°C according to the manufacturer's recommendations. The plasmid thus modified was purified on a QIAquick affinity column , then redigested with 80 units of HindIII for 1 h at 37°C. The 743 bp fragment corresponding to the promoter was separated on 0.8% agarose gel, then purified on a QIAquick affinity column.

– the cassette "*uidA*-IV2/*nos* term" was obtained by digesting 4 μg of the plasmid pMRT1092 with 40 units of HindIII and EcoRI for 1 h. The 2.2 kb fragment corresponding to the sequence *uidA*-IV2/*nos*

term was separated on 0.8% agarose gel, then purified on a QIAquick affinity column.

The ligation between the three fragments was carried out by mixing 100 ng of binary plasmid, 50 ng of promoter fragment and 50 ng of
5   the fragment corresponding to the "*uidA*-IV2/*nos* term" sequence in a reaction volume of 20 µl, in the presence of 2 µl of T4 10X DNA ligase buffer (New England Biolabs) and 400 units of T4 DNA ligase (New England Biolabs). The incubation was carried out in a thermocycle by subjecting the ligation mixture to 198 cycles each
10 consisting of a 30 sec incubation at 30°C, and a 30 sec incubation at 10°C. Previously prepared viable and competent Escherichia coli DH5α bacteria were transformed with half of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with tetracycline (12 mg/l), was extracted
15 according to the alkaline lysis method and analysed by enzymatic digestion and gene amplification using the desoxynucleotides 5' ATATGAGACTCTAATTGGATACCGAGGGG 3' selected from the transfer DNA of the binary plasmid and 5' TTGATTTCACGGGTTCGG 3' selected from the expression cassette in the *uidA* sequence. One of the retained
20 clones was designated pGA492MPr1092.

The plasmids pMRT1152, pMRT1171, pMRT1172 and pMRT1182 were transferred into a strain of *Agrobacterium tumefaciens* LBA4404 according to the technique described by Holsters *et al.* (1978). The plasmid DNA of the obtained clones, selected on LB media
25 supplemented with rifampicin (50 mg/l) and with tetracycline (5 mg/l), was extracted according to the alkaline lysis method, modified by adding lysozyme (25 mg/ml) to the cell resuspension buffer. The plasmid DNA obtained was analysed by enzymatic digestion and gene amplification using the desoxynucleotides 5'
30 ATATGAGACTCTAATTGGATACCGAGGGG 3' selected from the plasmid and 5' TTGATTTCACGGGTTGGG 3' selected from the expression cassette. The agrobacteria clones obtained were used to carry out plant genetic transformation.

35 **3.5. Production of the binary vectors pMRT1185, pMRT1186, pMRT1187 and pMRT1188 :**

The expression cassettes MPr1162/*uidA*-IV2/*nos* term,

MPr1164/*uidA*-IV2/*nos* term, MPr1165/*uidA*-IV2/*nos* term and
MPr1167/*uidA*-IV2/*nos* term were produced from plasmids pMRT1162,
pMRT1164, pMRT1165 and pMRT1167 respectively, and were cloned into
the binary plasmid pMRT1118 described previously.

5 A 10 µg amount of each of the plasmids pMRT1162, pMRT1164,
pMRT1165, and pMRT1167 were digested by PstI for 1 h at 37°C then
purified on an affinity column. The protruding 5' ends of these
various opened vectors were blunted using Pfu DNA polymerase
(Stratagene, La Jolla, USA) according to the supplier's

10 recommendations. The thus treated vectors were digested again
simultaneously by 20 units of EcoRI and 10 units of XmnI for 1 h
at 37°C. For each of these digestions, the DNA fragment
corresponding to the expression cassette was isolated on 1%
agarose gel and purified on a Qiaquick affinity column.

15 The ligation was carried out in a thermocycle (GeneAmp PCR Systems
9700) by mixing 100 ng of binary plasmid pMRT1118 prepared as
described above and 50 ng of expression cassette in a reaction
volume of 12 µl in the presence of 1.2 µl T4 10X DNA ligase buffer
(Epicentre Technologies), 1.2 µl of 25 mM ATP solution and 3 units

20 of 10X DNA ligase (Epicentre Technologies). The ligation reaction
consisted of a series of 200 identical cycles each consisting of a
30 sec step at 10°C and a 30 sec step at 30°C. Previously
prepared, viable and competent *Escherichia coli* DH5α bacteria were
transformed with half of the ligation reaction mixture. The

25 plasmid DNA from the clones obtained, selected on LB media
supplemented with kanamycin (50 mg/l), was extracted according to
the alkaline lysis method and analysed by enyzmatic digestion with
the enzymes BamH1 and EcoRI. The resulting clones were designated
pMRT1185, pMRT1186, pMRT1187, and pMRT1188, and contain

30 respectively the promoters MPr1162, MPr1164, MPr1165 and MPr1167.

3.6. Production of positive control binary vector pMRT1182.
The binary vector pMRT1182 was obtained by insertion of the PrD35S
CaMV promoter fragment and the *uidA*-IV2/term-nos sequence into the

35 binary plasmid pMRT1118 prepared as described above.
The PrD35S CaMV promoter was isolated by digesting 10 µg of the
plasmid pJIT163Δ successively with KpnI and HindIII for 1 h at

37°C. The 743 bp fragment corresponding to pD35S CaMV was isolated on 0,8 % agarose gel, then purified on a Qiaquick affinity column.

5   The "*uidA*-IV2/*nos* term" sequence was obtained by digesting 4 μg of plasmid pMRT1092 with 40 units of HindIII and EcoRI for 1 h. The 2.2 kb fragment corresponding to the "*uidA*-IV2/*nos* term" sequence was isolated on 0.8% agaros gel, then purified on a Qiaquick affinity column.

10   The ligation was carried out in the presence of 100 ng of binary plasmid, 50 ng of the PD35S CaMV fragment and 50 ng of the fragment corresponding to the *uidA*-IV2/term-nos sequence in a reaction volume of 20 μl, in the presencs of T4 (1X) DNA ligase buffer and 400 units of T4 DNA ligase (New England Biolabs). The incubation was carried out by PCR cycles in a «GeneAmp PCR System

15 9700» thermocycle as described previously. Previously prepared, vaible and competent *Escherichia coli* DH5α bacteria were transformed with half of the ligation reaction mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with kanamycin (50 mg/l), was extracted according to

20 the alkaline lysis method and analysed by enzymatic digestion. The resulting plasmid was designated pMRT1182.

3.7. Production of binary vectors pMRT1245, pMRT1246, pMRT1247, pMRT1248, pMRT1249, pMRT1250, pMRT1251, pMRT1252, and pMRT1253 :

25 The expression cassettes MPr1116/*uidA*-IV2/*nos* term, MPr1154/*uidA*-IV2/*nos* term, MPr1162/*uidA*-IV2/*nos* term, MPr1163/*uidA*-IV2/*nos* term, MPr1164/*uidA*-IV2/*nos* term, MPr1165/*uidA*-IV2/*nos* term, MPr1167/*uidA*-IV2/*nos* term, MPr1168/*uidA*-IV2/*nos* term and MPr1169/*uidA*-IV2/*nos* term were

30 produced from plasmids pMRT1116, pMRT1154, pMRT1162, pMRT1163, pMRT1164, pMRT1165, pMRT1167, pMRT1168 and pMRT1169 respectively, and were cloned into the HpaI site of the binary vector pMRT1195. A 10 μg amount of each of the plasmids pMRT1116, pMRT1154, pMRT1162, pMRT1163, pMRT1164, pMRT1165, pMRT1167, pMRT1168,

35 pMRT1169 was digested simultaneously by 20 units of PstI, 20 units of EcoRI and 10 units of XmnI for 1 h at 37°C. For each of these digestions, the corresponding DNA fragment to the expression

cassette was isolated on 1% agarose gel and purified on a Qiaquick affinity column. The 5' protruding ends of these different fragments were blunted using Pfu DNA polymerase (Stratagene, La Jolla, USA)according to the supplier's recommendations.

5 Ligation was carried out in a thermocycle (GeneAmp PCR Systems 9700) by mixing 100 ng of binary vector pMRT1195 prepared as described above and 50 ng of expression cassette in a reaction volume of 12 µl in the presence of 1.2 µl of T4 10X DNA ligase buffer (Epicentre Technologies), 1.2 µl of 25 mM ATP solution and 10 3 units of 10X DNA ligase (Epicentre Technologies). The ligation reaction consisted in a series of 200 identical cycles each consisting of a 30 sec step at 10°C and a 30 sec step at 30°C. Previously prepared, viable and competent *Escherichia coli* DH5α bacteria were transformed with half of the ligation reaction 15 mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with kanamycin (50 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion by the enzymes BamHI and EcoRI. The resulting clones were designated pMRT1245, pMRT1246, pMRT1247, pMRT1248, pMRT1249, 20 pMRT1250, pMRT1251, pMRT1252 and pMRT1253, and contain respectively the promoters MPr1116, MPr1154, MPr1162, MPr1163, MPr1164, MPr1165, MPr1167, MPr1168 and MPr1169.

3.8. Production of the positive control binary vector pMRT1254.
25 The binary vector pMRT1254 is used as a positive control during evaluation of the expression in plants.
The expression cassette MPr1092/*uidA*-IV2/*nos* term was cloned into the HpaI site of the binary vector pMRT1195 prepared as described above. It was obtained by digesting 10 µg of plasmid pMRT1182 with 30 40 units KpnI for 1 h at 37°C, purification of the digestion product on an affinity column and then digestion with 40 units of EcoRI. The 2.8 kb DNA fragment corresponding to the expression cassette was isolated on 1% agarose gel and purified on a Qiaquick affinity column. The 5' protruding ends sortantes of this fragment 35 were blunted with Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the supplier's recommendations.
Ligation was carried out in a thermocycle (GeneAmp PCR Systems

9700) by mixing 100 ng of the binary vector pMRT1195 prepared as described previously with 50 ng expression cassette in a reaction volume of 12 µl in the presence of 1.2 µl T4 10X DNA ligase buffer (Epicentre Technologies), 1.2 µl of 25 mM ATP solution and 3 units
5 of 10X DNA ligase (Epicentre Technologies). The ligation reaction consisted of a series of 200 identical cycles each consisting of a 30 sec step at 10°C and a 30 sec step at 30°C. Previously prepared, viable and competent *Escherichia coli* DH5α bacteria were transformed with half of the ligation reaction mixture. The
10 plasmid DNA of the obtained clones, selected on LB media supplemented with kanamycin (50 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion with the enzymes BamHI and EcoRI. The resulting clone was designated pMRT1254.

3.9. Production of the negative control binary vector pMRT1255.
The binary vector pMRT1255 is used as a negative control during evalutation of the expression in plants.
The expression cassette deprived of a promoter and corresponding
20 to the "*uidA*-IV2/*nos* term" sequence was cloned into the HpaI site of the binary vector pMRT1195, in order to use this plasmid as the negative control.
This sequence was obtained by digesting 10 µg of the plasmid pMRT1163 with 40 units of BamHI for 1 h at 37°C, purifiying the
25 digestion product on an affinity column and then digesting with 40 units of EcoRI at 37°C for 1 h. The 2.2 kb DNA fragment corresponding to the promoter-less expression cassette was isolated on 1% agaros gel and purified on a Qiaquick affinity column. The 5' protruding ends of this fragment were blunted using
30 Pfu DNA polymerase (Stratagene, La Jolla, USA) according to the supplier's recommendations.
Ligation was carried out in a thermocycle (GeneAmp PCR Systems 9700) by mixing 100 ng of binary vector pMRT1195 prepared as described above and 50 ng of expression cassette in a reaction
35 volume of 12 µl in the presence of 1.2 µl of T4 10X DNA ligase buffer (Epicentre Technologies), 1.2 µl of 25 mM ATP solution and 3 units of 10X DNA ligase (Epicentre Technologies). The ligation

reaction consisted of a series of 200 identical cycles each consisting of a 30 sec step at 10°C and a 30 sec step at 30°C. Previously prepared, viable and competent *Escherichia coli* DH5α bacteria were transformed with half of the ligation reaction
5   mixture. The plasmid DNA of the obtained clones, selected on LB media supplemented with kanamycin (50 mg/l), was extracted according to the alkaline lysis method and analysed by enzymatic digestion with the enzymes BamHI and EcoRI. The resulting clone was designated pMRT1255.
10

4. Measurement and comparison of the levels of expression of the different promoters according to the invention through transient expression experiments.
4.1. Culture and plant material production.
15 **4.1.1 In *vitro* culture of tobacco, leaf preparation.**
The transient expression experiments were carried out on tobacco leaves (*Nicotiana tabacum* L.) of the cultivar PBD6 aged 6 weeks. Mature tobacco cv. PBD6 seeds were sterilised for 10 min in a saturated calcium hypochlorite solution (70 g/l), then rinsed
20 thrice for 5 min in sterile deionized water. The sterile seeds were placed on MS20 media (Murashige et Skoog, 1962) and incubated for 6 weeks in a culture chamber (constant temperature of 24°C, photoperiod 16 h light / 8 h darkness, luminous intensity of 200 μmol photons.m^{-2}.sec^{-1}).
25 In order to avoid the foliar mesophyll cells splitting during transformation, the 2 main leaves of the tobacco plants PBD6 aged 6 weeks were excised from the plant 24 h before transformation with a gene gun, and placed, adlignous face up, on gentle plasmolysis BY3 media (Salts MS 4,4 g/l, myoinositol 100 mg/l,
30 thiamine 1 mg/l, KH$_2$PO$_4$ 200 mg/l, Saccharose 30 g/l, Sorbitol 45,5 g/l, 2,4 D 1 mg/l, pH 5,8).

4.1.2. Production and preparation of corn seed.
Transient expression experiments were carried out on the endosperm
35 of L2 corn seeds (cultivar SN 87 165), taken from corn plants cultured in a phytotron at 24°C, at 60% relative humidity and with a ephotoperiod of 16 h light / 8 h darkness.

Twelve days after pollinisation (12 DAP), the corn seed was taken and sterilised in a 20% bath of Domestos ®, with agitation for 5 min. Following the elimination of the Domestos ® by successive rinsing with deionized sterilised water, the pericarp and the
5   aleurone cell layer were carefully removed under sterile conditions. Tangential cuts of the now exposed endosperm were made and placed on filter paper soaked in the minimal Murashige et Skoog media (MS 5524, Sigma).

10 **4.1.3. Production of corn leaves.**
Transient expression experiments were carried out on leaves of L2 corn (cultivar SN 87 165), taken from the plant after two weeks culture in a phytotron at 24°C, at 60% relative humidity and with a photoperiod of 16 h light / 8 h darkness.
15 Twelve days after germination, the youngest leaves were taken and sterilised in a 20% bath of Domestos ®, with agitation for 5 min. The Domestos ® was eliminated by successive rinsing with deionized sterilised water, then the leaves were placed for 24 h onto the weak plasmolysis media N6P6 0.4M (salts MS 3.98 g/l, vitamines N6
20 100 mg/l, L-proline 700 mg/l, casein hydrosylate 100 mg/l, saccharose 20 g/l, sorbitol 36,4 g/l, mannitol 36,4 g/l, 2,4 D 1 mg/l, pH 5.8, phytagel 3g/l), adlignus face up, in order to avoid splitting of the foliar cells during transformation.

25 **4.2. Gold particle coating with the chimeric construction DNA.**
Biolistic transformation required prior deposition of DNA onto spherical gold beads of 0.6 μm in diameter that had been sterilised for 10 min in absolute ethanol (99.98%, with less than 0.02% water), washed four times in sterile deionized water, and
30 finally stored for a maximum of 4 weeks at -20°C in a solution of 50% glycerol.
The concentration of all of the control and test plasmids used during for the transformation experiments, was adjusted to 1 μg/μl. In each of the transformation experiments, an internal
35 reference control (pCaMV35Sluc) was cotransformed in order to normalise the variations of GUS activity between the different experiments (Leckie et al., 1994).

The coating of DNA onto the gold beads prepared as above was carried out in a sterile chamber under laminar flux. An aliquot fraction of 1.8 mg of sterile bead suspension in 30 µl of glycerol 50 %, was mixed vigorously in a vortex for 1 min, then for 10 sec

5 with 20 µl of DNA suspension containing 4 µg of one of the plasmids to be tested and 2 µg of the reference plasmid pCaMV35Sluc. Then, 20 µl of 2.5M CaCl2 were added an mixed vigorously for 10 sec. Next, 20 µl of 0.1M spermidine was added to the mixture and the whole mixture was agitated in a vortex for a

10 further 30 sec. The DNA coating of the beads was continued by incubating the mixture in ice for 15 min, then the coated beads were centrifuged at low velocity for 5 sec and washed twice in absolute ethanol.

After washing, the coated beads were resuspended in 32 µl absolute

15 ethanol, subjected to ultrasound treatment three times for a duration of 2 sec each time, vigorously mixed in a vortex for 15 sec, then immediately divided into 4 identical aliquot parts on sterile "macrocarrier" disks of the Biolistic PDS-1000/He system prepared according to the supplier's recommendations (BioRad,

20 Hercule, USA). The entire assembly of "macrocarrier support / macrocarrier bearing the bead deposit", was left to dry for 5 min.

4.3 Bombardment of tobacco foliar tissues and transient expression.

25 The bombardment of tobacco leaves was carried out using a Biolistic PDS-1000/He gene gun system by following the general recommendations of the supplier (BioRad, Hercule, USA) relating to the manipulations and assembly of the various components of the apparatus. Each leaf was bombarded twice successively under the

30 following shooting conditions :
- helium pressure selected for the acceleration of the coated gold beads was 6200 kPa (900 psi).
- the plant sample was placed at 9 cm from the bead acceleration zone.

35 - the shooting was carried out in a vacuum of 27 mm of mercury.
After bombardment the leaves were left in BY3 media and incubated

for 48 h in the dark in a culture chamber at 24°C. This incubation enabled transient expression of the transgenes introduced into the cells to occur.

4.3.1 Evaluation of the activity of the different promoters by histochemical staining.

The revelation of the expression of β-glucuronidase was carried out by histochemical staining as described by Jefferson et al. (1987). After 48 h in the culture chamber, each leaf was cut in two along the longitudinal axis of the central rib. Half of the leaf was incubated in staining buffer for β-glucuronidase (5-bromo, 4-chloro, 3-indolyl glucuronide (X-Gluc) 500 mg/l, Triton x100 0.05% in 0.1 M phosphate buffer, pH 7.0) for 48 h at 37°C, whereas the other half was frozen in liquid nitrogen, then stored at -80°C.

After staining, the leaves were bleached by dipping them in two 95% ethanol baths for respectively 3 and 12 h, then rinsed in distilled water and dried flat between two sheets of cellophane. The results of these histochemical stainings are presented in Figure V. The promoter activity of the various constructions was evaluated by the number of blue spots revealed on each leaf half after two shootings amounting to a total of 2 μg of DNA bearing the GUS reporter gene.

Two categories of promoters were identified. The leaves bombarded with the promoters MPr1116 and MPr1146 showed on average a number of blue spots significantly greater than 150 (Figure VIII) compared to the number of blue spots obtained by bombarding the leaves in the same conditions and with the same amount of reference control plasmid pMRT1092. The leaves bombarded with the promoters MPr1117 and MPr1147 show an average number of blue spots comprised between 50 et 150 (cf. Figure VIII).

To sum up, the chimeric promoters MPr1116 and MPr1146 enable or promote expression of β-glucuronidase to a level greater than or equal to that obtained using the strong constitutive reference promoter D35S prom.

4.3.2. Quantification of the expression of β-glucuronidase with the various promoters, by luminometric enzymatic activity determination.

The frozen leaf halves were ground in a mortar, then the powder
5   was left to thaw in extraction buffer (Tris Phosphate 25 mM pH
7.8, Dithiothréitol 2 mM, 1,2-diaminocyclohexane,
N,N,N',N'-tetracetic acid 2 mM, glycerol 10 %, Triton X100 1 %)
in a ratio of 1 ml of buffer to 200 mg of tissue. The mixture was
homogenised then incubated for 15 min in ice before being
10 clarified by centrifugation for 5 min at 16060 g.

GUS activity was measured on 20 µl of clarified crude leaf extract
using a "GUS-Light chemiluminescent reporter gene assay" detection
kit (Tropix Inc., Bedford, USA) according to the supplier's
recommendations. The measurement of light emission was carried out
15 using a Lumat LB 9507 luminometer (EGG-Berthold, Bad Wildbad,
Germany).

The luciferase activity was measured on 20 µl crude leaf extract
using a "Luciferase assay system" detection kit (Promega Corp.,
Madison, USA) according to the supplier's recommendations. The
20 measurement of light emission was carried out using a Lumat LB
9507 luminometer.

The results are presented in Figure VI. For each experiment (one
bombarded leaf = one crude extract), the ratio between
β-glucuronidase and luciferase activities measured by the
25 luminometer, was calculated. The average of the different
experiments for a given construction and the standard mean error
were determined. The results obtained show that :

The promoters MPr1116 and MPr1146 (Fig. IV) seem to significantly
increase expression over that of the double 35S CaMV reference
30 promoter (MPr1092, Fig. II). The promoter MPr1146 differs from
promoter MPr1116 by the insertion of a duplication of the "as-2"
box preceding the "as-1" box of the 35S CaMV promoter located
between the "as-1 like" box of the CoYMV promoter and the green
tissue specific element of the CsVMV promoter. This insertion of
35 elements from the 35S CaMV promoter in MPr1146 seems to slightly
increase the average degree of expression in relation to promoter

MPr1116 (4.5 %). These elements seem to be implicated in a positive synergy in such a combination.

The promoters MPr1116 and MPr1117 (Fig. IV) only differ with respect to the addition of an "as-1 like" box from the CoYMV
5   promoter in MPr1117. The average expression conferred by the promoter MPr1117 is significantly lower than that obtained with MPr1116 (22 %). This result suggests that the "as-1 like" element from CoYMV located in the 5' region of the chimeric promoter plays a repressor role in the promoter activity.

10   The promoter MPr1147 (Fig. IV) differs from promoter MPr1117 by the insertion of the "as-1" and "as-2" boxes between the "as-1 like" element from the CoYMV promoter and the green tissue specific element from the CsVMV promoter. The addition of these boxes and interaction with the other elements seems to lead to a
15   significant reduction in the average rate of expression with respect to MPr1117 (43 %). The association of these elements seems to favour a negative synergy. This can be used in constructs where the level of expression required in the transformed cell is relatively low, for example when providing antibiotic or herbicide
20   resistance for selection or marking purposes. Nevertheless, the weakest expression conferred by the promoter MPr1147 only differs by 51 % to that obtained by the promoter MPr1092. This negative effect could be explained in the case of the chimeric promoter, by a competition with trans-activating elements or else by a steric
25   hindrance effect of these factors on the promoter, thus diminishing its activity.

To conclude, the chimeric promoters MPr1116 and MPr1146 seem to cause an average expression of the GUS reporter gene in tobacco leaves that is noticeably better than that obtained by MPr1092.
30   This latter promoter is commonly reported in the literature as being the strongest chimeric promoter (of the order of 10 times greater than the strong constitutive promoter CaMV p35S (Kay et al., 1987), and routinely used for transgenesis. MPr1116 and MPr1146 can thus be classed among the strongest chimeric promoters
35   known to date.

The weaker expressing promoters can be of interest as mentioned above as promoters used for conferring antibiotic resistance for

the purposes of selection, for example in the same way as promoters of the "*nos*" type.

Figure IX illustrates complementary results. For each experiment, the ratio between β-glucuronidase activity and luciferase activity measured with the luminometer was calculated. The mean average of the different experiments for a given construction and standard mean error were determined. The results obtained show that :

     - the promoters MPr1163 and MPr1165 (cf. Figure VII), much as the promoters MPr1116 and MPr1146 (cf. Figure VII) are responsible for an average expression of the reporter gene slightly greater, by 8%, 5%, 6.5% and 11.5% respectively, than that obtained by the reference promoter MPr1092. The promoters MPr1163 and MPr1165 differ from the promoter MPr1116 by the insertion of respectively 2 and 4 series of as-2/as-2/as-1 boxes immediately downstream of the green tissue specific region from the CsVMV promoter. This insertion of multiple boxes from the CaMV 35S promoter into MPr1116 does not enable an increase in the average degree of expression with respect to that of promoter MPr1116.

     - the comparison of the average activity of the promoters MPr1146 and MPr1162 reveal that cloning into MPr1116 of the series of activating elements "as-2/as-2/as-1" from the CaMV promoter into the 5' region of the green tissue specific region of the CsVMV promoter, which is the case for MPr1146, is more favorable than cloning these same elements into the 3' region of this sequence, which is the case for MPr1162. This data indicates that the position of the activating elements with respect to each other is significantly related to the capacity of the promoter to transcribe efficiently. There is thus a synergy between the activating elements.

     - the comparison of the average activity of the promoters MPr1162, MPr1163 and MPr1165 reveals that multiplication of the as-2/as-2/as-1 boxes immediately downstream of the green tissue specific region of the CsVMV promoter does not provide a significant propotional increase in the activity of these promoters in transient expression. This data tends to show that there is no positive synergy between the whole of these activating element boxes assembled in such combinations. Without wishing to

be limited by theory, this could be explained by competition with trans-activating elements or else by steric hindrance problems of these trans-activating elements on the promoter.

5   - the reported negative effect of the "as-1 like" box from CoYMV that had been mentioned above during comparison of the activity of the promoters MPr1116, which has only one such box, and MPr1117, which has two such boxes, is confirmed. The deletion of these "as-1 like" boxes in promoter MPr1154 with respect to promoter MPr1147 enables a significant 65% increase in the

10   activity of the promoter.

      - the promoters MPr1162 and MPr1164 (Figure VII) only differ from the orientation of the series of as-2/as-2/as-1 boxes from CaMV downstream of the CsVMV sequence. No significant difference between these two promoters was observed. The orientation of these

15   boxes does not therefore appear, at least in the current configuration, to have any repercussions on the activity of the chimeric promoter.

To conclude, the chimeric promoters MPr1163 and MPr1165 seem to provoke an average expression of the GUS reported gene in tobacco

20   leaves at least as great as that obtained with the promoter MPr1092. The chimeric promoters MPr1163 and MPr1165, much as the promoters MPr1116 and MPr1146 can thus be classed among the strongest chimeric promoters described to date and can therefore be used routinely in transgenesis programs for dicotyledonous

25   plants, as a substitute to the CaMV D35S (double 35S or enhanced) promoter.

4.4. Corn bombardment and transient expression.

The bombardment of various corn tissues, and among others young

30   leaves and albumen, was carried out with a Biolistic PDS-1000/He gene gun system using the general recommendations of the supplier (BioRad, Hercule, USA) in relation to the manipulations and assembly of the different components of the apparatus. Each endosperm was bombarded twice in succession with tungsten

35   particles of 0.6 μm diameter, using the following shooting conditions :

      - the helium pressure to accelerate the particles was 6200 kPa

(900 psi),

- the plant sample was placed at 6 cm from the particle acceleration zone,

- the shooting was carried out in a vacuum of 27 mm of mercury.

5 Following bombardment, the endosperm were left in position and incubated for 24 h in the dark in a culture chamber at 26°C, to enable transient expression of the transgenes introduced into the cells.

10 **4.5. Evaluation of the activity of different promoters in corn endosperm by histochemical staining.**

Revelation of the expression of β-glucuronidase was carried out by histochemical staining as described by Jeffersson *et al.* (1987). After 24 h in a culture chamber, each portion of endosperm was 15 incubated for 48 h at 37°C in the presence of the substrate 5-bromo,4-chloro,3-indolyl glucuronide, X-Gluc at 500 mg/l in a 0.1 M phosphate buffer at pH 7.0 to which Triton x100 0.05% had been added.

After staining, the portions of endosperm were rinsed in water 20 then stored in a 96% ethanol bath.

The promoter activity of the different constructions were evaluated by the number of blue spots revealed on each portion of endosperm after two bombardments totalling 2 µg of DNA bearing the GUS reporter gene.

25 Analysis of the results of the histochemical stainings revealed that two categories of promoters could be identified. The endosperm bombarded with the promoters MPr1092, MPr1116, MPr1146 and MPr1147 showed on average relatively few blue spots of small diameter, ranging from 0 to 15 in number. The endosperm bombarded 30 with the promoters MPr1154, MPr1162, MPr1163, MPr1164, MPr1167 and MPr1169, and the reference control promoter MPr1218 showed a number of blue spots comprised between 10 and 30, the diameter of which was greater than those obtained with the above previously described promoters.

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4.6. Evaluation of β-glucuronidase expression in corn endosperm using luminometric enzyme assay measurement.

Frozen portions of endosperm were ground in a tube in extraction buffer (Tris Phosphate 25 mM pH 7.8, Dithiothreitol 2 mM, 1,2-diaminocyclohexane N,N,N',N'-tetracetic acid 2 mM, glycerol 10%, Triton X100 1%) using 1 ml of buffer for 200 mg of tissue. The mixture was homogenized then incubated for 15 min in ice before being clarified by centrifugation for 5 min at 16060 g. GUS activity was measured on 20 µl of clarified crude extract usig a "GUS-Light chemiluminescent reporter gene assay" detection kit (Tropix Inc., Bedford, USA) according to the supplier's recommendations. The measurement of light emission was carried out using a Lumat LB 9507 luminometer (EGG-Berthold, Bad Wildbad, Germany).

Luciferase activity was measured on 20 µl crude extract using a "Luciferase assay system" detection kit (Promega Corp., Madison, USA) according to the supplier's recommendations. The measurement of emitted light was carried out using a Lumat LB 9507 luminometer.

The results are presented in Figure VIII. For each crude extract, the relationship between β-glucuronidase activity and luciferase activity as measured by the luminometer, was calculated. The average of the different experiments for a given construction and standard mean error were determined. The results obtained show that the original chimeric viral promoter MPr1116 confers an activity 3.8 times greater than that obtained with the reference promoter MPr1218. This shows that the chimeric promoter, which contains "endosperm like" boxes possesses the necessary signals for expression of the GUS reporter gene in corn seed. The chimeric promoters MPr1162, MPr1163, MPr1164 and Mpr1165, which derive from promoter Mpr1116, have an activity comprised between 4.7 and 6 times that obtained with MPr1218 and between 1.2 and 1.6 times that obtained with MPr1116. Taking into account the fact that all of the chimeric promoters derived from MPr1116 possess the same basic sequence comprising the same regulatory elements or boxes apart from the activating elements from the CaMV promoter, the differences in activity observed seem to be due to the activating elements taken from CaMV.

The promoters containing a duplication of the "as-1 like" box from

CoYMV, which is the case for promoters MPr1167, MPr1168 and
MPr1169, all show on average an activity that is significantly
less than the promoters that only contain a single "as-1 like"
box, as is the case for promoters MPr1162, MPr1163, MPr1164 and
5 MPr1165. These results seem to confirm the repressor effect of
this element from CoYMV, already observed for transient expression
in tobacco. However, the comparison of average activities
conferred by the promoters MPr1116 and MPr1154 shows that the
absence of the "as-1 like" box and its replacement by an
10 activiating sequence "as-2/as-1" from CaMV in MPr1154, does not
lead to an increase in activity with respect to MPr1116. It would
therefore appear that it is the position of the boxes immediately
in the 5' region of the 104 bp sequence of CsVMV which is
unfavorable for the activity rather than the boxes themselves.
15 Without wishing to be bound by theory, it is likley that the
attachment of trans-activators in this position provokes a
conformational change in the whole of the "promoter sequence /
protein" assembly, and that this is unfavorable to the attachment
of other activating elements and/or the transcriptional machinery.
20 The analysis of the results reveals that the number of repetitions
of the combination of "as-2/as-2/as-1" boxes probably conditions
the activity of the chimeric promoter. For example, an increase in
the average activity between MPr1162, which possesses a single
series of elements, MPr1163, which has two such series, and
25 MPr1165, which has 4 such series, was observed, even if the
differences do not appear very significant. In the same way, the
activity increases between MPr1169, MPr1168 and MPr1167 which
respectively have 1, 2 and 3 series of boxes.
Finally, the orientation of these activating boxes or elements
30 does not seem to have any repercussions on the activity since no
significant difference was observed between the promoters MPr1162
and MPr1164.
To conclude, the chimeric promoters created according to the
present invention are functional and operational in
35 monocotyledonous plants and show in particular strong actiivity in
corn albumen. The promoters MPr1163 and MPr1165 are the promoters
that show the greatest activity of the GUS gene in transient

expression in corn endosperm 12 days after pollenisation. This activity is of a high level since it is about 6 times higher than that obtained with the strong promoter responsible for the active expression of gamma-zein, the major storage protein in corn albumen. These promoters can therefore be routinely used in transgenesis programs for monocotyledonous plants as an efficient alternative to other heterologous promoteurs.

5. Expression of the different promoters in tobacco after stable transformation.

5.1. Stable transformation of tobacco.

Tobacco transformation (*Nicotiana tabacum* L., cultivar PBD6) was carried out by infecting foliar disks isolated from tobacco plants aged 6 weeks with recombinant Agrobacteria according to the method described by Horsch *et al.* (1985).

During transformation, the Petri dishes were incubated in a culture chamber under the following conditions : temperature of 24°C, photoperiod of 16 h night / 8 h jour, luminous intensity of 200 µmol photons.m^{-2}.sec^{-1}, and apart from the initial coculture step, the whole of the callogenesis, regeneration and rooting steps were carried out on different selective media supplemented with Augmentin ® (400 mg/l) and kanamycin (200 or 100 mg/ml) ;

The various steps and media used are the following : - a coculture step lasting three days, during which the Agrobacteria infect the plant cells, on a solid MS30 coculture media (media with MS base (Murashige et Skoog, 1962) supplemented with vitamins (Gamborg *et al.*, 1968) 4.4 g/l (Sigma, M0404), Saccharose 30 g/l, agar 8 g/l (Merck), pH 5.7) supplemented with Benzyl Amino Purine at 1 mg/l and Indol-3 Acetic Acid at 0.1 mg/l .

- two bud formation steps lasting two weeks each in a culture chamber on a solid MS20 regeneration media (Salts and vitamins, MS 4.4 g/l (Sigma, M0404), Saccharose 20 g/l, agar 8 g/l (Merck), pH 5.7), supplemented with Benzyl Amino Purine at 1 mg/l, Indol-3 Acetic Acid at 0.1 mg/l, Augmentin ® at 400 mg/l et kanamycine at 200 mg/l.

- a development and rooting step lasting 3 weeks in a culture chamber on a solid MS20 development media supplemented with

Augmentin ® at 400 mg/l and kanamycine at 100 mg/l.

- a repotting step into glass pots in a culture chamber on a solid MS20 development media supplemented with Augmentin ® at 400 mg/l and kanamycine at 100 mg/l.

5.2. Measurement and comparison of β-glucuronidase activity in regenerated tobacco plants.

β-glucuronidase activity was measured on samples of first generation transgenic plants, taken at 2 weeks after their acclimatisation in a greenhouse. For each plant, three leaf samples were taken, one from an "aged" leaf (located the basal foliar level), one from a mature leaf (located at a median foliar level), and one from a young leaf (located at the plant apex). Each sample was ground in liquid nitrogen in a mortar and then the powder was resuspended in extraction buffer (Tris Phosphate 25 mM pH 7.8, Dithiothreitol 2 mM, 1,2-diaminocyclohexane, N,N,N',N'-tetracetic acid 2 mM, glycerol 10 %, Triton X100 1 %) in a ration of 1 ml of buffer for 200 mg of tissue. The mixture was homogenised then incubated for 15 min in ice before being clarified by centrifugation for 5 min at 16060 g.

GUS activity was measured on 20 µl clarified crude leaf extract using a "GUS-Light chemiluminescent reporter gene assay" detection kit (Tropix Inc., Bedford, USA) according to the supplier's recommendations. The measurement of light emission was carried out using a Lumat LB 9507 luminometer (EGG-Berthold, Bad Wildbad, Germany).

The quantity of total protein present in the crude extract was measured according to Bradford technique (1976), using a "BioRad protein assay" (BioRad, München, Germany).

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